

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
15 June 2006 (15.06.2006)

PCT

(10) International Publication Number  
**WO 2006/061207 A1**

(51) International Patent Classification:  
**G01N 33/543** (2006.01)

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(21) International Application Number:  
PCT/EP2005/013114

(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(22) International Filing Date:  
7 December 2005 (07.12.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
0426823.1 7 December 2004 (07.12.2004) GB

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

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**Published:**

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: SENSOR FOR DETECTION OF CARBOHYDRATE

(57) Abstract: A sensor for the detection or measurement of carbohydrate analyte (such as glucose) in fluid comprises components of a competitive binding assay the readout of which is a detectable or measurable optical signal (such as a FRET assay) retained by a material that permits diffusion of analyte but not the assay components, the assay components comprising: an animal lectin; and an analyte analogue capable of competing with analyte for binding to the lectin.



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SENSOR FOR DETECTION OF CARBOHYDRATE

The present invention relates to a sensor, to a method  
5 of preparing the sensor and to a method of using the sensor.

The sensor may be used in the measurement or monitoring  
of carbohydrate in fluid, for example glucose in body fluid,  
using optical techniques.

The sensor is particularly suitable for use in  
10 situations in which glucose levels must be closely monitored  
and/or where glucose measurements must be taken repeatedly,  
such as in diabetes management.

In the management of diabetes, the regular measurement  
of glucose in the blood is essential in order to ensure  
15 correct insulin dosing. Furthermore, it has been  
demonstrated that in the long term care of the diabetic  
patient better control of the blood glucose levels can  
delay, if not prevent, the onset of retinopathy, circulatory  
problems and other degenerative diseases often associated  
20 with diabetes. Thus, there is a need for reliable and  
accurate self-monitoring of blood glucose levels by diabetic  
patients.

It is desirable to measure blood glucose over the range  
of concentrations which may occur in a diabetic patient,  
25 that is, from 0 to 35 mM or even higher.

Currently, blood glucose is monitored by diabetic  
patients with the use of commercially available colorimetric  
test strips or electrochemical biosensors (e.g. enzyme  
electrodes), both of which require the regular use of a  
30 lancet-type instrument to withdraw a suitable amount of  
blood each time a measurement is made. On average, the  
majority of diabetic patients would use such instruments to

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take a measurement of blood glucose twice a day. However, the US National Institute of Health has recommended that blood glucose testing should be carried out at least four times a day, a recommendation that has been endorsed by the American Diabetes Association. This increase in the frequency of blood glucose testing imposes a considerable burden on the diabetic patient, both in financial terms and in terms of pain and discomfort, particularly in the long-term diabetic who has to make regular use of a lancet to draw blood from the fingertips. Thus, there is clearly a need for a better long-term glucose monitoring system that does not involve drawing blood from the patient.

There have been a number of proposals for glucose measurement techniques that do not require blood to be withdrawn from the patient.

It has been observed that the concentration of analytes in subcutaneous fluid correlates with the concentration of said analytes in the blood, and consequently there have been several reports of the use of glucose monitoring devices which are sited in a subcutaneous location. The use of competitive binding assays for glucose which can be remotely interrogated is of particular interest.

A method of assaying a competitive binding is to use a proximity-based signal generating/modulating moiety pair (discussed in US 6232120), which is typically an energy transfer donor-acceptor pair (comprising an energy donor moiety and an energy acceptor moiety). The energy donor moiety is photoluminescent (usually fluorescent).

In such methods, an energy transfer donor-acceptor pair is brought into contact with the sample (such as subcutaneous fluid) to be analyzed. The sample is then illuminated and the resultant emission detected. Either the

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energy donor moiety or the energy acceptor moiety of the donor-acceptor pair is bound to a receptor carrier, while the other part of the donor-acceptor pair (bound to a ligand carrier) and any analyte present compete for binding sites  
5 on the receptor carrier. Energy transfer occurs between the donors and the acceptors when they are brought together, which produces a detectable lifetime change (reduction) of the fluorescence of the energy donor moiety. Also, a proportion of the fluorescent signal emitted by the energy  
10 donor moiety is quenched.

The lifetime change is reduced or even eliminated by the competitive binding of the analyte. Thus, by measuring the apparent luminescence lifetime, for example, by phase-modulation fluorometry or time-resolved fluorometry (see  
15 Lakowicz, Principles of Fluorescence Spectroscopy, Plenum Press, 1983, Chapter 3), the amount of analyte in the sample can be determined.

It is to be noted that the efficiency of the energy transfer depends on the quantum yield of the donor, the  
20 overlapping of the emission spectrum of the donor with the absorption spectrum of the acceptor, and the relative distance and orientation between the donor and the acceptor.

In EP0561653 a method of interrogating a receptor and a ligand as described above, is disclosed.

25 An example of donor-acceptor energy transfer is fluorescence resonance energy transfer (Foerster resonance energy transfer, FRET), which is non-radiative transfer of the excited-state energy from the initially excited donor (D) to an acceptor (A). The donor typically emits at shorter  
30 wavelengths, and its emission spectrum overlaps with the absorption spectrum of the acceptor. Energy transfer occurs without the appearance of a photon and is the result of

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long-range dipole-dipole interactions between the donor and acceptor.

The term resonance energy transfer (RET) is more correct because the FRET process does not involve the appearance of a photon. However, FRET and RET are often used interchangeably.

An important characteristic of FRET is that it occurs over distances comparable to the dimensions of biological macromolecules. The distance at which FRET is 50% efficient, called the Förster distance, is typically in the range of 20-60 Å. Förster distances ranging from 20 to 90 Å are convenient for competitive binding studies.

Labelling an analyte-binding moiety with a donor (D) and an analyte analogue with an acceptor (A), or vice versa, would create an assay capable of generating a measurable response based on the donor-to-acceptor distance. Thus, binding of the D-"analyte-binding moiety" to A-"analyte analogue" results in a decrease in donor intensity or lifetime. The analyte in the sample competes for the analyte-binding moieties on D-"analyte-binding moiety", releasing D-"analyte-binding moiety" from the acceptor (A). The intensity decay time and phase angles of the donor are thus expected to increase with increasing glucose concentration.

These principles have been used in glucose sensing by energy transfer.

WO91/09312 describes a subcutaneous method and device that employs an affinity assay based on glucose (incorporating an energy transfer donor-acceptor pair) that is interrogated remotely by optical means. Examples WO97/19188, WO00/02048, WO03/006992 and WO02/30275 each

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describe glucose sensing by energy transfer, which produce an optical signal that can be read remotely.

A person skilled in the art will appreciate that the acceptor could be a fluorophore. Any fluorescent signal emitted by the energy acceptor moiety following excitation with a beam of incident radiation at a wavelength within the absorption spectrum of the energy acceptor moiety is unaffected by the FRET process. It is therefore possible to use the intensity of the fluorescent signal emitted by the energy acceptor moiety as an internal reference signal, for example in continuous calibration of the sensor or to monitor the extent to which the sensor has degraded and thus indicate the need to implant or inject a fresh sensor. The fall of this signal below an acceptable baseline level would indicate the need to implant or inject a fresh sensor.

The energy acceptor moiety may, however, be a non-fluorescent dye. In this case a compound with fluorescence quenching capability is used instead of the specific energy acceptor moiety. An example of a powerful and non-specific fluorescence quencher is given by Tyagi et al. Nature Biotechnology (1998) **18**: p49.

The systems discussed above rely on Concanavalin A (Con A) as the glucose binding moiety. Concanavalin A is a lectin. The term "lectin" includes any carbohydrate-binding protein not obviously involved in carbohydrate metabolism and which does not belong to any of the major classes of immunoglobulins. Lectins show selective binding to carbohydrates via carbohydrate recognition domains (CRDs). Lectins occur naturally in both monomeric and multimeric forms, the latter often comprising a number of subunits, each bearing several CRDs.

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Concanavalin A is not stable for long periods under assay conditions. Experiments by the inventors (see Example 8) suggest that Concanavalin A is stable at body temperature for less than 20 days.

5        Also, Concanavalin A is toxic and potentially immunogenic (however, it is used in glucose assays in small quantities which are thought to be safe in the human body).

It has also been suggested that sweet pea and lentil lectins could be used as glucose binding moieties in such systems ("A Potentially Implantable Fluorescent Glucose  
10        Sensor Based on Molecular Recognition in Poly(ethylene glycol) Hydrogels", Ryan J. Russell et al., presented to American Institute of Chemical Engineers). However, these lectins are expected to have similar disadvantages to Con A.

15        US 6232130 discloses an assay in which low valency lectins ("carbohydrate binding ligands") are used. These have 3 or fewer CRDs. The assay uses an analyte analogue ("glycoconjugate") comprising a carbohydrate, a label (e.g. a FRET component) and a carrier molecule. The carrier  
20        molecule may be a protein (e.g. bovine serum albumin, BSA) or a synthetic polymer.

The present inventors have appreciated that there is a need to find carbohydrate binding moieties which have good stability and which do not have the disadvantages associated  
25        with Con A. They have investigated the use of alternative carbohydrate binding moieties. Surprisingly, they have found that animal lectins, including human lectins, can be used as carbohydrate binding moieties.

Accordingly, in a first aspect, the present invention  
30        provides a sensor for the detection or measurement of carbohydrate analyte in fluid, the sensor comprising components of a competitive binding assay the readout of

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which is a detectable or measurable optical signal retained by a material that permits diffusion of the analyte but not the assay components, the assay components comprising:

an animal lectin; and

5        an analyte analogue capable of competing with carbohydrate for binding to the lectin.

Preferably, the analyte is a monosaccharide. In a preferred embodiment, the analyte is glucose.

Preferably, the sensor is suitable for the detection or  
10       measurement of glucose in body fluid, for example subcutaneous fluid. It is desirable for the sensor to be suitable for use *in vivo*, and this is discussed in more detail below.

Preferably, the analyte analogue is capable of competing  
15       with glucose at physiological calcium concentrations. Typical physiological calcium concentrations are in the range of 1.15 to 1.29 mM.

#### *Detection*

20       Suitable detection techniques include FRET, fluorescence energy transfer, fluorescence polarisation, fluorescence quenching, phosphorescence, luminescence enhancement, luminescence quenching, diffraction or plasmon resonance.

25       The binding assay generating the optical signal should preferably be reversible such that a continuous monitoring of fluctuating levels of analyte can be achieved. This reversibility is a particular advantage of the use of a binding assay format in which the components of the assay  
30       are not consumed.

Preferably, the detectable or measurable optical signal is generated using a proximity based signal



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generating/modulating moiety pair. A signal is generated or modulated when a first member of the pair is brought into close proximity with a second member of the pair.

Preferably, the proximity based signal  
5 generating/modulating moiety pair is an energy donor moiety and energy acceptor moiety pair. Energy donor moieties and energy acceptor moieties are also referred to as donor and acceptor chromophores respectively. An energy acceptor which does not emit fluorescence is referred to as a  
10 quenching moiety.

In this case, the lectin is labelled with one of an energy donor and energy acceptor moiety pair and the analyte analogue is labelled with the other of the energy donor and energy acceptor moiety pair.

15 The most preferred embodiment of the sensor of the invention incorporates an assay which generates an optical readout using the technique of FRET.

Where the assay is to be used *in vivo*, it is desirable for donors to fluoresce at 550 to around 700 nm and for  
20 acceptors to absorb light at around 650 nm. This avoids overlap between the donor fluorescence and *in vivo* autofluorescence at lower wavelengths.

Alexa Fluor 594™ (e.g. as succinimidyl ester) is an energy donor moiety with a suitable emission spectrum for  
25 use *in vivo*. This dye absorbs at 594 nm and fluoresces at 620 nm.

The HMCV dyes described in WO05/059037 are suitable energy acceptor moieties for use in the invention. These dyes are stabilised carbenium ions. An example is Hexa-  
30 Methoxy-Crystal Violet succinimidyl ester (HMCV-1).

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Alternatively, QSY 21™ may be used as an energy acceptor moiety with Alexa Fluor 594™ as an energy donor moiety.

Fluorescence lifetime or fluorescence intensity measurements may be made. Fluorescence lifetime may be measured by phase modulation techniques (discussed below).

In a preferred embodiment, the lectin is labelled with AlexaFluor 594 as energy donor moiety, the analyte analogue is labelled with HMCV-1 as energy acceptor moiety, and fluorescence lifetime is measured by phase modulation techniques.

The material retaining the assay components preferably provides sufficient space for the energy donor and the energy acceptor moieties to separate when not bound to one another so that energy transfer can cease.

#### *Lectin*

Preferably, the lectin provides a stable signal in the assay for at least 10 days, more preferably for at least 14 days. It is particularly preferable that a stable signal is provided when the sensor is implanted in the human body.

Surprisingly, the present inventors found that MBL was stable in a glucose assay for at least 17 days (see Example 8). Earlier accounts reported a biological half-life for MBL of 4-7 days (Kilpatrick (2002) Transfus. Med. 12, 335).

Preferably, the lectin is a C-type (calcium dependent) lectin.

Preferably, the animal lectin is a vertebrate lectin, for example a mammalian lectin, more preferably a human or humanized lectin. However, it may alternatively be a bird lectin, fish lectin or an invertebrate lectin such as an insect lectin.

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Suitably, the lectin is a human lectin derived from the human body. Alternatively, the lectin may be a recombinantly manufactured lectin.

As a further alternative, the lectin may be a humanised animal lectin, for example a humanised bovine lectin. This applies where there is a corresponding human lectin. The lectin may be humanised in an analogous way to antibodies.

Suitably, the lectin is in multimeric form. Multimeric lectins may be derived from the human or animal body. Alternatively, the lectin may be in monomeric form. Monomeric lectins may be formed by recombinant methods or by disrupting the binding between sub-units in a natural multimeric lectin derived from the human or animal body. Examples of this are described in US 6232130.

Preferably, the lectin has three or more CRDs. More preferably, the lectin has 6 or more CRDs.

Preferably, the lectin is a collectin (collagen-like lectin). These are C-type animal lectins which have collagen like sequences (Gly-Xaa-Yaa triplet). MBL is a C-type collectin whereas Concanavalin A is a C-type lectin. Monomeric collectin CRDs can be prepared by the action of collagenase.

Preferably, the lectin is mannose binding lectin, conglutinin or collectin-43 (e.g. bovine CL-43) (all serum collectins) or a pulmonary surfactant protein (lung collectins).

Mannose binding lectin (also called mannan binding lectin or mannan binding protein, MBL, MBP), for example human MBL, has proved particularly interesting. MBL is a collagen-like defence molecule which comprises several (typically 3 to 4 (MALDI-MS), though distributions of 1 to 6 are likely to occur (SDS-PAGE)) sub-units in a "bouquet"

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arrangement, each composed of three identical polypeptides. Each sub-unit has a molecular weight of around 75 kDa, and can be optionally complexed with one or more MBL associated serine proteases (MASPs). Each polypeptide contains a CRD.

5 Thus, each sub-unit presents three carbohydrate binding sites. Trimeric MBL and tetrameric MBL (which are the major forms present in human serum, Teillet et al., Journal of Immunology, 2005, page 2870-2877) present nine and twelve carbohydrate binding sites respectively.

10 MBL occurs naturally in the body as part of the innate immune system where it binds mannose moieties coating the surface of bacteria. Human MBL is not toxic and is non-immunogenic to humans. MBL of other species is expected to be immunogenic but not toxic to humans.

15 Human MBL is commercially available both in a form derived from the human body and in a recombinantly manufactured form. It is used as a replacement therapy in the treatment of MBL deficient patients who are believed to have increased susceptibility to infectious diseases.

20 Suitably, the lectin is MBL substantially in trimeric and/or tetrameric form. As explained above, trimeric MBL and tetrameric MBL are believed to be the major naturally occurring multimeric forms in human serum.

Alternatively, the lectin may be a pulmonary surfactant  
25 protein selected from SP-A and SP-D. These proteins are similar to MBL. They are water-soluble collectins which act as calcium dependent carbohydrate binding proteins in innate host-defence functions. SP-D also binds lipids. SP-A has a "bouquet" structure similar to that of MBL (Kilpatrick DC  
30 (2000) Handbook of Animal Lectins, p. 37). SP-D has a tetrameric "X" structure with CRDs at each end of the "X".

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Other suitable animal lectins are those set out in the following list:

- PC-lectin (US 20030216300, US 20040265898)
- CTL-1 (US 179528/10)
- 5 • Keratinocyte membrane lectins (Parfuemerie und Kosmetik **74**, 164-80)
- CD94 (Eur J Immunol **25**, 2433-7)
- P35 (synonym: human L-ficolin, a group of lectins) (Immunol Lett 67, 109-12)
- 10 • ERGIC-53 (synonym: MR60) (Mol Biol Cell, 7, 483-93)
- HIP/PAP (Eur J Biochem **267**, 1665-71)
- CLECSF8 (Eur J Immunol **34**, 210-20)
- DCL (group of lectins) (Appl no 00231996/US)
- GLUT family proteins, especially GLUT1, GLUT4 and
- 15 GLUT11 (PNAS **97**, 1125-30)

Further suitable animal lectins are set out in Appendices A, B and C of "Handbook of Animal Lectins: Properties and Biomedical Applications", David C. Kilpatrick, Wiley 2000.

- 20 The lectin is preferably labelled as discussed above. More preferably, the lectin is labelled with an energy donor moiety.

#### *Analyte Analogue*

- 25 Preferably, the analyte analogue comprises a plurality of carbohydrate or carbohydrate mimetic moieties which bind to binding sites of the lectin. The term "carbohydrate" includes sugars.

- 30 Suitable carbohydrate mimetic moieties include peptides such as keratin peptide (SFGSGFGGGY) which mimics N-acetyl glucosamine. It has been shown that keratin peptide can

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inhibit MBL (Mantacta et al. 2001 J. Immunol. 166, 4148-4153).

It has been found by the inventors that the affinity of common carbohydrate moieties for MBL is as follows:

5 D-Mannose, *N*-acetyl-D-mannosamine, D-fructose, D-leucrose, erlose, *N*-acetyl-D-glucosamine, L-Fucose > *myo*-inositol, D-glucose, D-arabinose, D-palatinose, D-turanose, D-sorbitol, D-ribose, D-tagatose > D-lyxose > lactose, L-arabinose, D-galactose.

10 Whilst they do not wish to be bound by this theory, the inventors believe that strong binding to MBL and other lectins is the result of binding at a number of sites. The binding at each site is relatively weak (low affinity) but the cumulative effect is strong binding (high avidity).

15 Thus, an analyte analogue which does not bind all the binding sites is more readily displaced by analyte, which binds all the binding sites, than an analyte analogue which does bind all the binding sites. This explains why an analyte analogue containing mannose, which has a higher  
20 affinity for MBL than does glucose, can be displaced by glucose.

The parameters which affect avidity of an analyte analogue for a given lectin are:

- 25 - number of carbohydrate or carbohydrate mimetic moieties;
- affinity of the carbohydrate or carbohydrate mimetic moieties for the lectin;
- calcium concentration (at least for MBL); and
- flexibility of the analyte analogue.

30 Physiological calcium concentration cannot be controlled. However, the other parameters can be selected to give an analyte analogue with an appropriate measurement

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range. The effect of analyte analogue flexibility on assay performance has not previously been identified or addressed.

Previously disclosed analyte analogues (e.g. those of US 6232130) have comprised globular proteins to which

5 carbohydrate and energy donor or energy acceptor moieties are conjugated. In such molecules the carbohydrate and energy donor or energy acceptor moieties have fixed positions. This means that the analyte analogues cannot necessarily adopt a conformation which allows binding of a plurality of

10 carbohydrate moieties to lectin CRDs.

Also, the relative positioning of the carbohydrate and energy donor or energy acceptor moieties in such analyte analogues may not allow optimum interaction between the energy donor and acceptor moieties when the analyte analogue

15 and lectin are bound. This will affect FRET and weaken the optical signal.

Finally, these analyte analogues often do not bind to lectins at physiological calcium concentrations. The calcium concentration required for optimum binding of mannose

20 glycoconjugates to MBL has been found to be around 20 mM.

These insights have been used by the inventors to develop various analyte analogues which have different avidity for the same lectin, and which can therefore be used to measure carbohydrate concentration over different ranges.

25 Preferably, the assay is capable of measuring blood glucose for concentrations over at least part of the range of 0 to 35 mM glucose, for example over the range of 0 to 25 mM glucose. Suitably, the  $IC_{50}$  value is around 15 mM glucose. More preferably, the assay is capable of measuring

30 glucose concentrations over the range of 2 to 10 mM glucose. A dosage-response curve which is as close as possible to linear within this range is desirable.

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Three different types of structure for the analyte analogue are of particular interest.

Carbohydrate-Protein Conjugate or Carbohydrate-Dendrimer

5 Conjugate

First, the analyte analogue may be a carbohydrate-protein conjugate or a carbohydrate-dendrimer conjugate. In either of these cases, carbohydrate mimetic moieties may be used instead of or in addition to carbohydrate moieties.

10 Examples of suitable carbohydrates for use in such conjugates are monosaccharides and oligosaccharides.

Suitable monosaccharides are optionally derivatised tetroses, pentoses, hexoses, heptoses or higher homologous aldoses or ketoses, for example optionally derivatised D-glucose, D-mannose, N-acetyl-D-glucosamine, L-fucose, D-fructose, D-tagatose or D-sorbitol.

Suitable oligomers may be linear or branched homooligomers or mixed oligomers, for example containing from 2 to 50 carbohydrate units.

20 The preferred glycosylation is 1→6 or 1→2, as 1→3 and 1→4 glycosylation is expected to interrupt MBL binding. For example, nona(1→6)-α-glucose (dextran 1500 Da) is expected to have higher avidity for MBL than 1,3-β-D-glucoses (e.g. laminanarihexaose). Suitable oligosaccharides include  
25 pannose, maltose, maltotriose, isomaltotriose, D-leucrose, erlose, D-palatinose, D-turanose or 1 to 250 kDa dextran (preferably 1 to 40 kDa dextran, for example 1 kDa, 1.5 kDa, 5 kDa, 6 kDa, 10 kDa, 12 kDa, 20 kDa, 25 kDa or 40 kDa dextran).

30 Preferably, the analyte analogue comprises at least one carbohydrate moiety selected from D-fructose, D-leucrose, N-acetyl-glucosamine, D-mannose, L-fucose, N-acetyl-

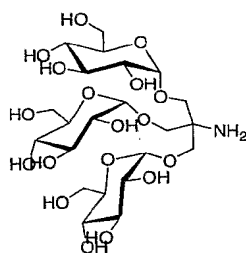


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mannosamine, D-arabinose, *myo*-inositol, D-tagatose, erlose, D-glucose, D-palatinose, D-turanose, D-ribose, D-sorbitol.

More preferably, the analyte analogue comprises at least one glucose moiety and/or at least one N-acetyl glucosamine moiety and/or at least one mannose moiety, since these have a high affinity for MBL and other animal lectins. It is believed that these moieties bind to binding sites of the lectin via their C3 and C4 hydroxyl groups.

Examples of a synthetic branched saccharide are dendrimer "wedges" used to construct dendrimers (e.g. TRIS derived trisaccharide with an amine linker, shown below). Such "wedges" could be conjugated onto a protein such as HSA (human serum albumin), for example via a bifunctional amine linker.



Preferred proteins for use in the conjugate are human proteins having a molecular weight of at least 10 kDa, preferably at least 20 kDa. Preferably, the protein has a non-globular overall tertiary structure. It is believed that this assists binding at more than one binding site, leading to high avidity. Monoclonal antibodies such as herceptin and Remicade™ (an immunoglobulin having several globular domains with a non-globular "Y"-shaped overall tertiary structure) are suitable. Other alternative suitable proteins are human thrombin, human lactoferrin and Factor XIII.

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As another example of a carbohydrate-protein conjugate, the protein may be a lectin-derived protein, for example a lectin with the CRDs removed.

Suitably, the conjugate may be a carbohydrate-albumin conjugate. For example, the conjugate may be a mannose-HSA conjugate or a mannose-BSA (bovine serum albumin) conjugate. However, conjugates of this type are not preferred since, as mentioned above, binding to MBL has been found to be dependent on calcium concentration. At physiological calcium concentrations a 70 kDa mannose-HSA conjugate with 20 mannose residues was found not to bind MBL. The dependence on calcium concentration decreases with increasing mannosylation.

The skilled person would be aware of synthetic routes to conjugates of this type. As an example, N-isothiocyanato-4-aminophenyl-O- $\alpha$ -D-mannopyranoside (Man-ITC) can be conjugated onto HSA.

Dendrimers for use in the invention preferably have amine-functionalised, carboxylic acid-functionalised or hydroxyl-functionalised surfaces. Preferably, the dendrimers are of the polyamidoamine (PAMAM) or polypropylenimine (DAB) type. Preferably, the molecular weight is less than 60 kDa, for example around 2 to 10 kDa. Such dendrimers can be cleared by the kidney (Kobayashi et al., 2004, J. Mag. Reson. Imaging 20(3) 512-518).

#### Polysaccharide

Second, the analyte analogue may be an optionally derivatised polymer of carbohydrate and/or carbohydrate mimetic moieties (both included in the term "polysaccharide" used herein). Dextran (a glucose polymer, poly(1 $\rightarrow$ 6)- $\alpha$ -glucose) binds strongly to MBL and similar lectins. The

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inventors believe that this is a result of the large number of glucose residues (approximately 430 residues in 70 kDa dextran) and the flexibility of dextran. The concentration of glucose needed to displace dextran from MBL is therefore  
5 high.

A glucose assay based on dextran and MBL can optimally measure glucose concentrations of around 30 mM. This is much higher than the normal 5 mM glucose concentration in blood. Such an assay can measure glucose concentrations  
10 from 0 to 10 mM with a sensitivity of only about one third of the total phase response (0.25 ° Phase shift per mM Glc, see Example 7).

The present inventors therefore looked for alternative analyte analogues which would bind MBL and similar lectins  
15 less strongly, so that more than one third of the total phase response would be available in the 0 to 10 mM glucose range.

The inventors discovered that treating dextran with periodate (which oxidatively cleaves the glucose pyranose  
20 ring between the 2 and 3 or 3 and 4 carbons to form a dialdehyde) can be used to reduce the avidity of dextran for MBL and similar lectins. This appears to be because MBL binds to the 3 and 4 equatorial hydroxyls of glucose as explained above. The 3 and 4 hydroxyl groups could  
25 inactivated in other ways (for example by oxidation, reduction, alkylation, substitution, glycosylation or esterification).

Very surprisingly, the inventors found that periodate treated dextran-MBL binding is not prevented by  
30 physiological calcium concentrations. This is in contrast to mannose-HSA conjugate MBL binding as discussed above. It would have been expected that periodate-treated dextran MBL

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binding was prevented by physiological calcium concentrations, particularly since the glucose moieties of dextran have lower affinity for MBL than do mannose moieties.

5           Theoretically two equivalents of periodate per glucose unit could be consumed (one per diol). However, it has been found that 1 to 100 equivalents of periodate is suitable for 70 kDa dextran.

          Treatment of the dialdehyde with ammonia or an amine  
10 followed by reduction (e.g. with sodium cyanoborohydride) can be used to give an aminated dextran. A procedure can also be used in which the dialdehyde is aminated followed by optional catalytic hydrogenation to yield the free amine. Benzylamine is a useful amine in this context as the  
15 intermediate before hydrogenation is a dextran derivative with lipophilic moieties. Also, a benzylamine derived aminated dextran can be used to assess the degree of periodate cleavage using spectrophotometric techniques. If the benzyl group is removed by catalytic hydrogenation,  
20 energy donor or energy acceptor moieties can be coupled to the remaining amine.

          Alternatively, a polysaccharide-based analyte analogue can be synthesised which bears different carbohydrate or carbohydrate mimetic moieties of different affinity for MBL  
25 and similar lectins. Derivatisation of dextran with mannose moieties to adjust the glucose detection range in a Concanavalin A FRET assay is disclosed in Ballerstadt et al., Diabetes Technology & Therapeutics, vol. 6, no. 2, 2004.

30           Galactose binds to MBL with very low affinity. Therefore, an analyte analogue containing galactose moieties

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(for example galactose-derivatised dextran) has lower avidity for MBL than the underivatised analyte analogue.

N-acetyl-glucosamine has a high affinity for MBL. Therefore, an analyte analogue containing N-acetyl-glucosamine moieties (for example GlcNAc derivatised amylose) would have higher avidity for MBL than the underivatised analyte analogue.

Preferably in this embodiment, the analyte analogue is selected from optionally derivatised dextran, mannan, amylose, amylopectin, glycogen, hyaluronate, chondroitin, heparin, dextrin, inulin, xylan, fructan and chitin. As galactose has very low affinity for MBL, a non-derivatised polymer of galactose such as agarose is not preferred as an analyte analogue.

The skilled person would be aware of ways in which a polysaccharide can be derivatised with carbohydrate moieties. As an example, amine-functionalised polysaccharides (for example aminodextran, which is commercially available from CarboMer, San Diego, California, USA, Cat. No. 5-00060 or Molecular Probes, Eugene, Oregon, USA, Cat No. D1862) or the aminated dextrans referred to above may conveniently be derivatised. Alternatively, alcohol groups in the polysaccharide and amine groups in the carbohydrate or carbohydrate mimetic moieties may be linked using divinylsulphone. Methods of derivatising dextran are disclosed in EP 594772.

Examples of suitable carbohydrate moieties for derivatisation of polysaccharides are those set out in connection with carbohydrate-protein and carbohydrate-dendrimer conjugates above.

Synthetic Polymer

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Third, the analyte analogue may be a synthetic polymer.

Synthesis of an artificial polymer rather than derivatisation of a protein or polysaccharide allows the parameters of the polymer (for example molecular flexibility, water solubility, molecular weight, nature of carbohydrate or carbohydrate mimetic moieties, number of carbohydrate or carbohydrate mimetics moieties, number of proximity based signal generating/modulating moieties) to be readily controlled to improve assay performance. Compared with a polysaccharide, a synthetic polymer has the advantage that the number of carbohydrate moieties can be controlled independently of the length of the polymer. Furthermore, using non-ring containing monomers such as 2-hydroxyethyl acrylate (HEA) in the polymer gives increased molecular rotational flexibility compared with dextran.

Without wishing to be bound by this theory, the inventors believe that it is important that proximity based signal generating/modulating moieties are close to the binding moiety to generate a strong signal. Globular ligands concentrate binding moieties and proximity based signal generating/modulating moieties on a spherical surface so that they are close. In dextran, which is linear, the backbone consists of binding moieties, and consequently it is not possible to control whether binding is close to or remote from a proximity based signal generating/modulating moiety. This can be controlled in the synthetic polymer by positioning the binding moieties close to the proximity based signal generating/modulating moieties.

Preferably in this embodiment, the analyte analogue is a non-saccharide flexible water-soluble polymer bearing pendant carbohydrate or carbohydrate mimetic moieties.

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The term "flexible" includes polymers which are capable of significant intermonomeric rotation. Preferably, the polymers do not contain bulky groups (for example ring structures, tert-butyl groups or other sterically large groups) other than the pendant carbohydrate or carbohydrate mimetic moieties and proximity based signal generating/modulating moieties. Preferably, such polymers have very few double bonds in the backbone structure (for example less than 10 %). Suitably, such polymers do not have a globular tertiary structure, although they may have such a structure.

Preferably, the polymer is unbranched (unlike the dendrimers discussed above). This improves flexibility of the polymer. However, the polymer may be branched or cross-linked to some extent provided that this does not lead to formation of a hydrogel. For example, 1 to 5 branchings in a polymer with an overall molecular weight of 100 kDa is acceptable.

The term "water soluble" includes polymers having a water solubility at room temperature of at least 4 mg/ml, preferably at least 25 mg/ml, more preferably at least 50 mg/ml, for example at least 100 mg/ml. The solubility will be higher at body temperature. It is important that the polymer is water soluble so that it will dissolve in interstitial fluid when used in a sensor in the body as discussed below. The polymer should be water soluble even when bound to a carbohydrate binding molecule such as MBL.

Preferably, the polymer includes no more than 1 to 5 types of monomer unit, more preferably no more than 3 monomer units.

Suitably, the polymer is a co-polymer comprising first monomer unit residues bearing pendant carbohydrate or

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carbohydrate mimetic moieties and second monomer unit residues bearing pendant proximity based signal generating/modulating moieties. Alternatively or additionally, a single monomer unit residue bearing both  
5 pendant carbohydrate or carbohydrate mimetic moieties and pendant proximity based signal generating/modulating moieties may be used. The use of first and second monomer units is preferred, since the amounts of carbohydrate or carbohydrate mimetic moieties and proximity based signal  
10 generating/modulating moieties can then be controlled independently.

Preferably, the co-polymer is a random co-polymer. However, it may also be an alternating co-polymer. Use of a block co-polymer with large blocks is not preferred.  
15 However, a block co-polymer with blocks of low molecular weight (for example 1 to 3 kDa) may be used.

Preferably, when used in an assay with MBL as a carbohydrate binding molecule, the polymer binds to MBL at 0 mM glucose at least 50 % as strongly as aminodextran, more  
20 preferably at least as strongly as aminodextran, but is more easily inhibited. It is particularly desirable that the polymer is easily inhibited (large proportion of total phase response) over the range of 0 to 35 mM glucose, and especially over the range of 2 to 15 mM. This provides an  
25 assay over glucose concentrations of particular physiological interest which is more sensitive than a similar assay using aminodextran as a glucose analogue.

More than one type of monomer unit residue bearing carbohydrate or carbohydrate mimetic moieties may be  
30 present. The carbohydrate or carbohydrate mimetic moieties may be different, with different affinities for MBL and similar lectins.



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Suitably, the first monomer units (or single monomer units) are each a double bond-containing derivative of a carbohydrate or carbohydrate mimetic moiety. However, the first monomer units (or single monomer units) may each be a double bond-containing molecule containing a functional group to which the carbohydrate or carbohydrate mimetic moiety can be linked, suitably after polymerisation.

Preferably, the double bond-containing derivative of the carbohydrate or carbohydrate mimetic moiety is an allyl or vinyl containing derivative of a carbohydrate or carbohydrate mimetic moiety. Other suitable double bond-containing derivatives of carbohydrate or carbohydrate mimetic moieties include homologues of allyl derivatives, for example 3-butenyl or 4-pentenyl derivatives, or styrene derivatives with the carbohydrate or carbohydrate mimetic moiety at the 4 position. Further suitable double bond-containing derivatives of carbohydrate or carbohydrate mimetic moieties include HEA, 2-hydroxyethyl methacrylate (HEMA) or vinyl alcohol (VA) based derivatives.

The carbohydrate or carbohydrate mimetic moieties may be linked to amine, acid, alcohol and/or sulphone functional groups of the first monomer units (or single monomer units). For example, alcohol groups in the monomer units and amine groups in the carbohydrate or carbohydrate mimetic moieties may be linked using divinylsulphone. Where the carbohydrate is mannose, the linkage should not be via the C3-OH or C4-OH groups, since these are important in binding to MBL. In this case, divinylsulphone linkage may be inappropriate.

Amino derivatised carbohydrate moieties can be produced by reductive amination of disaccharides. This allows the carbohydrate moiety to be linked at its anomeric position (C1).

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The carbohydrate or carbohydrate mimetic moiety could be connected to alcohol groups (e.g. in HEA) by Fischer glycosidation.

It is not necessary for the first monomer units (or  
5 single monomer units) to contain double bonds.

Examples of suitable carbohydrates for use in the copolymer are as discussed in connection with Carbohydrate-Protein Conjugates above.

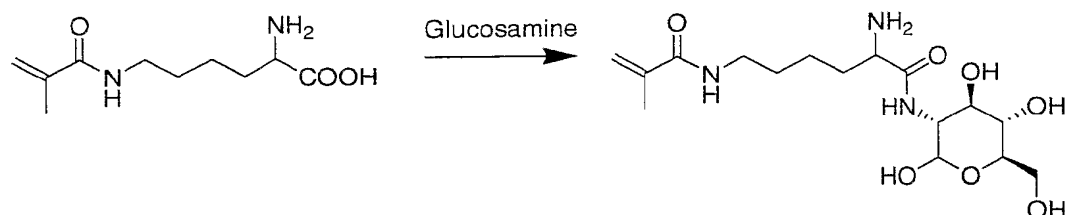
Suitably, the second monomer units (or single monomer  
10 units) are each a double bond-containing molecule containing a functional group to which the proximity based signal generating/modulating moiety can be linked, suitably after polymerisation. Suitable functional groups include acid, alcohol and/or sulphone. Linkage after polymerization helps  
15 to minimize loss of the expensive proximity based signal generating/modulating moieties.

However, the second monomer units (or single monomer  
units) may contain the proximity based signal generating/modulating moieties. In this case, the  
20 discussion above of suitable polymerisable groups and linkages applies.

In a preferred embodiment, the second monomer units are each N-(3-aminopropyl)methacrylamide or a derivative thereof.

25 In a preferred embodiment, the single monomer units are each a double bond containing, carbohydrate or carbohydrate mimetic moiety containing derivative of lysine. An example is shown below (multistep reaction scheme):

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The starting material in this reaction scheme is methacryloyl-L-lysine, available through Polysciences Europe (Eppelheim, Germany). After polymerization, the alpha amine group could be linked to the proximity based signal generating/modulating moiety.

Preferably, the polymer further contains third monomer unit residues which do not bear pendant carbohydrate or carbohydrate mimetic or proximity based signal generating/modulating moieties. This helps to increase flexibility.

Flexibility is increased by using third monomer units which are sterically unhindered such as HEA. Flexibility is also increased by using third monomer units which are uncharged. A polymer containing no third monomer units would have a large number of positively charged ammonium groups which would need to be inactivated to minimize decreased flexibility because of electrostatic repulsion.

More than one type of third monomer can be included in the polymer.

Preferably, the third monomers units are each a double bond-containing molecule containing a hydrophilic group, for example a hydroxyl group. It is not preferred for the third monomers units to be a lipophilic double bond-containing molecule, for example styrene.

In a preferred embodiment, the third monomer units are each HEA, vinyl pyrrolidone, MMA, HEMA, vinyl alcohol and/or

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ethylene glycol. However, the skilled person will appreciate that there are many other double bond-containing molecules containing hydrophilic groups which could be used.

Suitably, the monomer units are reacted by addition  
5 polymerization. The addition polymerization may be free-radical initiated, for example using potassium peroxodisulfate (PPS) or another peroxide compound.

Other possibilities are condensation polymerization (for example ionic condensation polymerization), ring opening  
10 polymerization and atom transfer radical polymerization (ATRP). The skilled person will appreciate that the nature of the monomer units will depend on the desired method of polymerization (for example double bond containing monomer units are not necessary for condensation polymerization).

15 Suitably, the monomer units are mixed before initiator is added.

Preferably, the polymerization reaction takes less than two days. The length of the polymerization can be used to control the molecular weight of the co-polymer product.

20 Suitably, the polymerization reaction takes place under oxygen-free conditions.

Suitably, the polymerization reaction is carried out at room temperature.

Where no single monomer units are used, the first monomer  
25 units are preferably present in the reaction mixture in an amount of 20 to 70 wt%, more preferably in an amount of 30 to 50 wt%.

Where the third monomer units are used, they are preferably present in the reaction mixture in an amount of 5  
30 to 15 wt%.

It will be appreciated that the composition of the polymer does not exactly reflect the amounts of monomer

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units present in the reaction mixture. This is because of the influence of other factors (for example steric hindrance and solubility).

5 It should also be noted that the analyte analogue may consist of two or more separate entities which together act as an analyte analogue. In particular, the analyte analogue may consist of a first entity with at least two analyte analogue moieties and a second entity which is an analyte  
10 binding molecule such as a lectin. For example, acceptor labelled MBL and donor labelled MBL can be used together with unlabelled dextran or unlabelled synthetic polymer as a template to bring the donor labelled MBL and acceptor labelled MBL in proximity of each other so that FRET occurs.  
15 (example using Con A given by Gestwicki *et al.* (2002) *Chemistry and Biology* **9**, p163).

The analyte analogue is preferably labelled with one or more proximity based signal generating/modulating moieties as discussed above. Preferably, the analyte analogue  
20 comprises one or more energy acceptor moieties (for example HMCV-1 or Alexa Fluor 594™, discussed above). However, it may also comprise one or more energy donor moieties.

The proximity based signal generating/modulating moieties may be attached to the analyte analogue as  
25 discussed in connection with the carbohydrate or carbohydrate mimetic moieties above. For example, labelling of dextran can be achieved by direct divinylsulphone coupling or by amination (as described above) followed by coupling. Where an amine derivatised dextran is used as the  
30 analyte analogue, care must be taken to avoid cross linking during attachment of the proximity based signal generating/modulating moieties, as this could lead to

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undesirable precipitation. Methods of derivatising dextran with DVS in order to minimize cross-linking are discussed in EP594772.

The analyte analogue should have a molecular weight  
5 high enough to prevent escape from the sensor but low enough that precipitation does not occur when the analyte analogue binds to the lectin. Analyte analogues having a weight in the range of 25 to 250 kDa, more preferably 40 to 250 kDa, more preferably still 70 to 150 kDa, highly preferably 100  
10 to 120 kDa, for example 110 kDa are preferred. Analyte analogues based on 110 kDa dextran are particularly preferred.

Optionally, the analyte analogue and lectin are tethered together.

15

#### *Sensor Construction*

Preferably, the components of the assay are retained by a material which has a pore size that permits diffusion of analyte but not the assay components. However, this  
20 selectivity may be achieved in other ways, for example by using a material which allows diffusion of uncharged materials.

Preferably, the components of the assay are retained by a shell or matrix material. The analyte analogue and/or  
25 lectin may be grafted onto this material. More preferably, the material is biodegradable as described in WO00/02048. Optionally, the sensor may comprise small particles retained by a shell of biodegradable material as described in WO03/006992.

30 In a preferred embodiment, the components of the assay are retained by a shell of biodegradable material encapsulating the assay components whilst allowing glucose

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to contact the assay components, and the biodegradable material comprises a co-polymer having hydrophobic and hydrophilic units, as described in WO2005/110207.

One or more assay component chambers may be present  
5 within the shell.

Preferably, the co-polymer is a random copolymer.

Preferably, the co-polymer has a permeability of at least  $5.0 \times 10^{-10} \text{ cm}^2/\text{s}$ .

The word "permeability" is used to refer to the overall  
10 permeability of analyte (glucose) through hydrated co-polymer which can be measured experimentally.

Preferably, once implanted in the body the co-polymer degrades over a period of one week to one year, for example 30 days. For a typical polymer thickness of 5  $\mu\text{m}$  this  
15 corresponds to a degradation rate of 0.17  $\mu\text{m}/\text{day}$ .

Preferably, for mobility of glucose, the biodegradable material has a molecular weight cut-off limit of no more than 25000 Da. More preferably, the biodegradable material has a molecular weight cut-off limit of no more than 10000  
20 Da.

Preferably, the weight fraction of the hydrophobic units is from 10 to 90 % of the co-polymer, more preferably from 10 to 50 % of the co-polymer.

Preferably, the molecular weight of each hydrophilic  
25 unit is from 200 to 10000 Da, more preferably from 400 to 4000 Da.

Preferably, the hydrophilic units of the co-polymer each comprise an ester of polyethylene glycol and a diacid. As an alternative to polyethylene glycol, a mixed polymer of  
30 ethylene glycol and propylene glycol may be used, and/or the polyether backbone may be substituted with hydrophobic and/or hydrophilic groups. As a further alternative to

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polyethylene glycol, poly-tetrahydrofuran (poly-THF) may be used.

Preferably, the hydrophilic units comprise terephthalic acid and/or succinic acid as diacids. Other suitable  
5 diacids are oxalic acid, tartaric acid, phthalic acid, aspartic acid, malonic acid and oligomeric or polymeric diacids, for example poly(dimer acid-sebacic acid). In one preferred embodiment, the diacid is terephthalic acid only. In an alternative preferred embodiment, the molar ratio of  
10 terephthalic acid to succinic acid is 1:2 to 2:1, suitably 1:1.

Alternatively, the hydrophilic units of the co-polymer may comprise oligomers. Suitable oligomers are oligomers of hydroxyethylmethacrylate (HEMA), vinylpyrrolidone, vinyl  
15 alcohol, carbohydrates, ethylene oxide and/or 2-acrylamido-2-methyl propane sulfonic acid. Where the hydrophilic units comprise HEMA, biodegradable linkages (for example ester linkages such as terephthalate linkages) are provided within the polymer to increase biodegradability.

20 Preferably, the molecular weight of each hydrophobic unit is from 400 to 5000 Da.

Preferably, the hydrophobic units of the co-polymer comprise an ester of butane-1,4-diol and a diacid. As an alternative to butane-1,4-diol, pentane-1,5-diol or hexane-  
25 1,6-diol may be used.

Preferably, the hydrophobic units comprise terephthalic acid and/or succinic acid as diacids. In a preferred embodiment, the molar ratio of terephthalic acid to succinic acid is 1:2 to 2:1, suitably 1:1. Alternatively, the  
30 hydrophobic units comprise terephthalic acid only as diacid. Other suitable diacids are given above.



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Alternatively, the hydrophobic units of the co-polymer can comprise oligomers of methylmethacrylate (MMA), polyurethane and/or amides (for example Nylon-6, oligo-N-tertiary butylacrylamide or oligo-N-isopropylacrylamide).

5 Where the hydrophobic units comprise MMA, biodegradable linkages (for example ester linkages such as terephthalate linkages) are provided within the polymer to increase biodegradability.

Preferred polymers have the general formula  
10 aPEG(T/S)bPB(T/S)c where "a" denotes the molecular weight of the PEG chain, "b" the weight fraction of the PEG(T/S) (polyethylene glycol terephthalate/succinylate) in the resulting polymer and "c" the weight fraction of the PB(T/S) (polybutylene terephthalate/succinylate) in the resulting  
15 polymer. Examples of such polymers are 600PEGT80PBT20, 1000PEGT80PBT20, 2000PEGT80PBT20, 4000PEGT80PBT20, 1000PEGT50PBT50 and 1000PEG(T/S)60PB(T/S)40(T/S 50%). The polymers are biodegradable, have high glucose permeability and have molecular weight cut-off properties at around 25000  
20 Da.

Some of these polymers are disclosed in US6383220 and EP1247522.

The envelope of co-polymer preferably has a thickness of 1 to 50  $\mu\text{m}$ .

25 In a second aspect, the present invention relates to a method of preparing a sensor as described herein.

Chemical methods for the preparation of polymer microcapsules include phase separation (coacervation), solvent evaporation and/or extraction.

30 Suitable physical methods for the preparation of polymer microcapsules include spray drying, spray coating, spray chilling, rotary disk atomisation, fluid bed coating,

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coextrusion (for example stationary nozzle coextrusion, centrifugal head coextrusion, or submerged nozzle coextrusion) and pan coating.

## 5 *Sensor Use*

In a third aspect, the present invention relates to a method of detecting glucose using a sensor as described herein, comprising implantation of the sensor into the skin of a mammal, detection or measurement of glucose using  
10 external optical means.

In a fourth aspect, the present invention relates to a method of detecting glucose using a sensor as claimed described above, comprising detection or measurement of glucose using external optical means by illumination of a  
15 said sensor present in or below the skin of a mammal.

Preferably, the method further comprises degradation of biodegradable material in the sensor.

The sensor may be introduced within the skin by injection, preferably using a syringe, or by other methods,  
20 in particular by any method described in W000/02048. The sensor is preferably of a size suitable for injection through a narrow gauge needle to minimise the discomfort to the patient. Preferably, the sensor has a maximum dimension of 20  $\mu\text{m}$  to 1 mm. However, a rod-shaped sensor having a  
25 larger maximum dimension may be used.

The sensor may be introduced within the thickness of the dermis, or subdermally, or may be introduced to the epidermis, although in the latter case it would be likely to be expelled from the skin by outgrowth of the epidermal  
30 layers, possibly before the biodegradable material has degraded.

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Because the sensor is located within the skin, an optical signal generated in the sensor is preferably detected transcutaneously (i.e. through the higher layer(s) of the skin) thus obviating the need for any direct  
5 connection between the sensor and the external environment which may lead to infection.

However, detection may alternatively take place via a hollow or transparent means (for example a needle or optical fibre) which allows the sensor to be illuminated by external  
10 optical means without passing light through the skin.

Once the sensor is in place in a cutaneous location glucose measurements can be taken as often as is necessary with no adverse effects. This is a particular advantage in relation to the long-term care of diabetic patients because  
15 if glucose measurements are taken more frequently, tighter control can be maintained over the level of glucose in the blood and the risk of developing conditions related to poorly regulated blood glucose, such as retinopathy, nephropathy, neuropathy, general micro- and macrovascular  
20 damage and poor circulation, will be reduced.

Because the sensor of the invention does not itself contain any of the optical components required to interrogate the readout of the assay (these being preferably provided separately and located outside the body) the sensor  
25 can easily be provided in a form which is injectable with minimal discomfort to the patient.

Sensors incorporating an assay employing the technique of FRET may be interrogated by supplying incident radiation at a wavelength within the absorption spectrum of the energy  
30 donor moiety and measuring the intensity of the emitted fluorescence or the lifetime of the excited state. Commonly known methods are:

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1. Steady state measurement
2. Time-domain lifetime measurement
  - a. Single photon counting
  - b. Streak camera
  - 5 c. Gated detection (pulse sampling)
  - d. Up-conversion
3. Frequency domain lifetime measurement
  - a. Phase-modulation fluorometry (heterodyne detection)
  - 10 b. Phase sensitive detection (homodyne detection)

Further description of the principles may be found in Lakowicz, J. R. "Principles of Fluorescence Spectroscopy, Second Edition", 1999.

The preferred method for interrogating the assay is  
15 phase-modulation fluorometry.

A suitable optical set-up for interrogating the assay (Fig. 6) consists of a light-emitting diode (LED) 11, which emits light within the emission spectrum of the energy donor moiety. The LED is operated by a driver circuit 13, which  
20 modulates the LED at a frequency which results in a sufficient phase shift, preferably in the range of 45°. For a fluorophore with a lifetime of 3 ns, the preferred frequency is 50 MHz. The light emitted by the LED is filtered by an excitation filter 15 and directed towards the  
25 sensor 16 by a dichroic beam splitter 17 and focused onto the sensor/skin above the injected sensor 16 by a lens 19. The fluorescence emitted by the sensor is collected by the lens 19. The light passes through the dichroic beam splitter and is filtered through an emission filter 21. The filtered  
30 light is focused by a lens 23 onto the detector 25, in this case an avalanche photodiode (APD). The APD is reverse biased by an APD bias supply 27, which is controlled by a

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signal processing and control unit 29. The signal from the APD is amplified by a trans-impedance amplifier 31, filtered by a bandpass filter 33 and sampled by a first analog-to-digital converter (ADC) 35. Correspondingly, the modulated drive signal to the LED is sampled by a second ADC 37. The signal sampled on the first ADC 35 is:

$$Y_1(t) = A_1 \sin(2\pi f t + \varphi_{f1} + \varphi_1)$$

$A_1$  is the amplitude of the detected signal from the assay,  $f$  is the modulation frequency,  $\varphi_{f1}$  is the phase lag introduced by the donor fluorophore and  $\varphi_1$  is a fixed phase lag introduced by the electronic and optical set-up.

The signal sampled on the second ADC 37 is:

$$Y_2(t) = A_2 \sin(2\pi f t + \varphi_2)$$

$A_2$  is the amplitude of the modulated drive signal to the LED and  $\varphi_2$  is a fixed phase lag introduced by the electronic set-up

The signal processing and control unit derives the phase lag  $\varphi_{f1}$  introduced by the energy donor moiety by comparing the two sampled signals and compensating for the fixed and known phase lags introduced by the electronics and optics.

Measurements are taken by holding the fluorometer close to the skin and in alignment with the sensor. The phase lag is converted to analyte concentration by the use of a phase-to-analyte-calibration function, such as

$$\text{analyte concentration} = A + Bx / (k + x),$$

where  $A$  is the phase at no analyte present,  $B$  is the phase at maximal response,  $x$  is the measured phase, and  $k$  is the

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dissociation constant between the receptor and the analyte analogue.

An alternative measurement technique is measurement of fluorescence intensity.

5        In this case, the optical means should supply a first beam of incident radiation at a wavelength within the absorption spectrum of the energy donor moiety and preferably a second beam of incident radiation at a wavelength within the absorption spectrum of the energy  
10    acceptor moiety (this applies where the energy acceptor moiety is also a fluorophore). In addition, the optical means should preferably be capable of measuring optical signals generated in the sensor at two different wavelengths; wavelength 1 within the emission spectrum of  
15    the energy donor moiety (the signal generated in connection with the measurement of analyte) and wavelength 2 in the emission spectrum of the energy acceptor moiety (which could be the analyte signal or the internal reference or calibration signal).

20        The fluorometer separately measures the following parameters:

At wavelength 1 (energy donor moiety)

Excitation light intensity,  $I(1,0)$

Ambient light intensity,  $I(1,1)$

25        Intensity of combined fluorescent and

ambient light,  $I(1,2)$

At wavelength 2 (energy acceptor moiety)

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Excitation light intensity,  $I(2,0)$ Ambient light intensity,  $I(2,1)$ Intensity of combined fluorescent and  
ambient light,  $I(2,2)$ 

5 Again, measurements are taken by holding the fluorometer close to the skin and in alignment with the sensor. When making transcutaneous measurements of the fluorescent signals generated in the sensor it is necessary to take account of the absorption of signal by the skin.

10 The absorptivity of human skin is found by experiment to be lowest in the range from 400 nm to 900 nm. The final output provided is the normalised ratio between the fluorescent intensity from the two fluorophores, defined by the following relation (Equation 1):

15 
$$\text{Final output} = (I(1,2) - I(1,1)) * I(2,0) / (I(2,2) - I(2,1)) * I(1,0)$$
  
(1)

The final output from the optical means (e.g. the fluorometer) as given by Equation 1 above is converted to analyte concentration preferably by means of a computer

20 using calibration data which can be obtained based on the principles set out in WO00/02048.

#### *Further Aspects of Invention*

In a fifth aspect, the present invention relates to a

25 sensor for the detection or measurement of carbohydrate analyte in fluid, the sensor comprising components of a competitive binding assay the readout of which is a detectable or measurable optical signal retained by a

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material that permits diffusion of analyte but not the assay components, the assay components comprising:

a lectin; and

5 an analyte analogue comprising optionally derivatised dextran in which the 3- and/or 4- hydroxyl groups of at least one of the glucose units have been inactivated, the analyte analogue being capable of competing with analyte for binding to the lectin. Preferably, the dextran is periodate-treated dextran.

10 In a sixth aspect, the present invention relates to a sensor for the detection or measurement of carbohydrate analyte in fluid, the sensor comprising components of a competitive binding assay the readout of which is a detectable or measurable optical signal retained by a  
15 material that permits diffusion of analyte but not the assay components, the assay components comprising:

a lectin; and

an analyte analogue comprising a mannose-protein conjugate capable of competing with analyte for binding  
20 to the lectin.

Preferably, the mannose-protein conjugate is one prepared using a molar ratio of mannose to HSA in the range of 10:1 to 150:1, for example 15:1, 30:1, 60:1 or 120:1.

In a seventh aspect, the invention relates to a sensor  
25 for the detection or measurement of a carbohydrate analyte in fluid, the sensor comprising components of a competitive binding assay the readout of which is a detectable or measurable optical signal retained by a material that permits diffusion of the analyte but not the assay  
30 components, the assay components comprising:



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a carbohydrate binding molecule labelled with one of a proximity based signal generating/modulating moiety pair; and

5 a carbohydrate analogue capable of competing with the analyte for binding to the carbohydrate binding molecule, the carbohydrate analogue being a flexible water-soluble polymer comprising:

10 polymerized residues of first monomer units, the first monomer unit residues bearing pendant carbohydrate or carbohydrate mimetic moieties and pendant moieties which are the other of the proximity based signal generating/modulating moiety pair; and/or

15 co-polymerised residues of second monomer units and third monomer units, the second monomer unit residues bearing pendant carbohydrate or carbohydrate mimetic moieties and the third monomer unit residues bearing pendant moieties which are the other of the proximity based signal generating/modulating moiety pair.

20 In an eighth aspect, the invention relates to a method of producing a polymer as described above, comprising one of the following procedures:

25 a) polymerising monomer units each bearing a pendant carbohydrate or carbohydrate mimetic moiety and a pendant proximity based signal generating/modulating moiety and optionally third monomer units;

30 b) co-polymerising first monomer units each bearing a pendant carbohydrate or carbohydrate mimetic moiety and second monomer units each bearing a pendant proximity based signal generating/modulating moiety and optionally third monomer units;

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- 5 c) polymerising monomer units each bearing a pendant carbohydrate or carbohydrate mimetic moiety and a pendant functional group for linking to an proximity based signal generating/modulating moiety and optionally third monomer units, then reacting the monomer unit residues with the proximity based signal generating/modulating moieties;
- 10 d) co-polymerising first monomer units each bearing a pendant carbohydrate or carbohydrate mimetic moiety and second monomer units each bearing a pendant functional group for linking to an proximity based signal generating/modulating moiety and optionally third monomer units, then reacting the second monomer unit residues with the proximity based signal
- 15 generating/modulating moieties;
- 20 e) polymerising monomer units each bearing a pendant functional group for linking to a carbohydrate or carbohydrate mimetic moiety and a pendant different functional group for linking to an proximity based signal generating/modulating moiety and optionally third monomer units, then reacting the monomer unit residues with the carbohydrate or carbohydrate mimetic moieties and proximity based signal generating/modulating moieties; or
- 25 f) co-polymerising first monomer units each bearing a pendant functional group for linking to a carbohydrate or carbohydrate mimetic moiety and second monomer units each bearing a pendant different functional group for linking to an proximity based signal
- 30 generating/modulating moiety and optionally third monomer units, then reacting the first monomer unit residues with the carbohydrate or carbohydrate mimetic

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moieties and the second monomer unit residues with proximity based signal generating/modulating moieties.

Features described in connection with any aspect of the invention can be applied to other aspects of the invention.

5

The invention will be further illustrated with reference to examples, and to the Figures in which:

Fig. 1 shows the glucose dose response from a human MBL and dextran assay system for various dextran molecular weights (Example 6).

Fig. 2 shows the glucose dose response from (a) a human MBL and 110 kDa dextran assay system and (b) a Con A and 110 kDa dextran assay system (Example 8).

Fig. 3 shows the glucose dose response from a human MBL and HSA mannose ELLA assay system (Example 11);

Fig. 4 shows the glucose dose response from a human MBL and periodate-treated dextran ELLA assay system (Example 11);

Fig. 5 shows the glucose dose response from a human MBL and 70 kDa dextran assay system (Example 15).

25

Fig. 6 shows a suitable optical set-up for interrogating the assay.

### Examples

30

### General

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The following materials were used:

p-Aminophenyl- $\alpha$ -D-mannopyranosyl isothiocyanate, Bovine  
serum albumin- $\alpha$ -D-mannopyranosyl isothiocyanate (23 eq Man  
5 pr. BSA), Human serum albumin, sodium periodate, Biotin-N-  
hydroxy succinimide, o-phenylene dihydrochloride,  
benzylamine, ammonia, sodium cyanoborohydride  
(Sigma-Aldrich).

10 Nunc F96 MaxiSorp plate (Nunc, Denmark).

PD-10 columns, Streptavidin-HRP (Amersham bioscience).

Dextrans (Pharmacosmos, Denmark).

15

Mannan binding lectin (available from several sources e.g.  
Statens Serum Institute, Copenhagen, Denmark).

Dialysis tube Spectra/Por (Spectrum Laboratories Inc.,  
20 California, USA). Float-A-Lyzer™ 25.000 MWCO dialysis tubing  
was from Spectrum Laboratories Europe (Breda, The  
Netherlands).

Sorbitan monooleate (Span® 80), Azodiisobutyronitrile  
25 (AIBN) and 2-hydroxyethylacrylate were from Sigma-Aldrich.  
N-(3-aminopropyl) methacrylamide hydrochloride was from  
Polysciences Europe (Eppelheim, Germany). 2,2'-Azobis[2-(2-  
imidazolin-2-yl)propane] dihydrochloride (VA-044) was from  
Wako GmbH (Neuss, Germany).

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Allyl  $\alpha$ -D-Glucopyranoside and Allyl 2-acetamido-2-deoxy- $\alpha$ -D-glucopyranoside were from Glycon Biochemicals, Germany. Allyl  $\alpha$ -D-Galactopyranoside was from Sigma-Aldrich.

- 5    PBS is 20 mM Phosphate, 150 mM NaCl, pH 7.4, and TBS is 20 mM TRIS, 150 mM NaCl, 1,25 mM  $\text{CaCl}_2$ , pH 7.4 unless otherwise stated.

Abbreviations: MBL, Mannan Binding Lectin; PBS, Phosphate  
10    buffered saline; TBS, TRIS buffered saline; ELLA, Enzyme Linked Lectin Assay.

#### Example 1: Staining of MBL

- 15    Human MBL was buffer changed (by dialysis) to a 10 mM  $\text{NaHCO}_3$  buffer containing 150 mM NaCl and 1.25 mM  $\text{Ca}^{2+}$ , pH 8.7. The dye used for staining was Alexa Fluor™ 594 succinimidyl ester (AF594-SE) (Molecular Probes, Eugene, Oregon, USA). The dye was dissolved in dry DMSO and added slowly (10 min.)  
20    to the MBL in bicarbonate buffer. Reaction was allowed to take place for 1 hour. The staining was performed with 15 times molar excess (with respect to the polypeptide unit) of dye. Purification was carried out by dialysis against 10 mM Tris buffer pH 7.4, 150 mM NaCl and 1.25 mM  $\text{Ca}^{2+}$ . The  
25    obtained degree of labelling of the stained protein was determined by UV spectroscopy as 2.3 dyes per subunit of MBL.

#### Example 2: Preparation of Dextran

30

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150 kDa Dextran (6.00 g, 0.4  $\mu$ mol) was dissolved in 250 mM  $K_2HPO_4$  pH 11.5 (600 mL). Sodium borohydride (3 g, 0.08 mol) was added followed by the addition of divinylsulfone (15 ml, 0.15 mol). The reaction mixture was stirred for 30 min at  
5 RT, before neutralization to pH 7.2 with conc. HCl. After 30 min stirring, the resulting mixture was dialysed (MWCO 10-12 kDa) in water (3  $\times$  25 L). The contents were transferred to an Erlenmeyer flask and 24 % ammonia (200 mL) was added. After 2 h, the pH was adjusted to 10.5, and the reaction was  
10 stirred overnight. Excess ammonia was removed by dialysis (MWCO 10-12k) in water (8 $\times$ 25 L), and the entire contents were lyophilised to yield the aminodextran 5.75 g (78 %, based on an aminodextran MW of 185 kDa) as a white fluffy substance. Elemental analysis was used to make a rough  
15 estimate of the molecular weight, amine incorporation, and amount of incorporated divinylsulfone. (Found C 39.86; H 6.26; N 0.16; S 3.08 % Dextran 150k, ~22 DVS-NH<sub>2</sub>, ~160 DVS-OH, and ~720 H<sub>2</sub>O requires C 39.55; H 6.60; N 0.16; S 3.07 %).

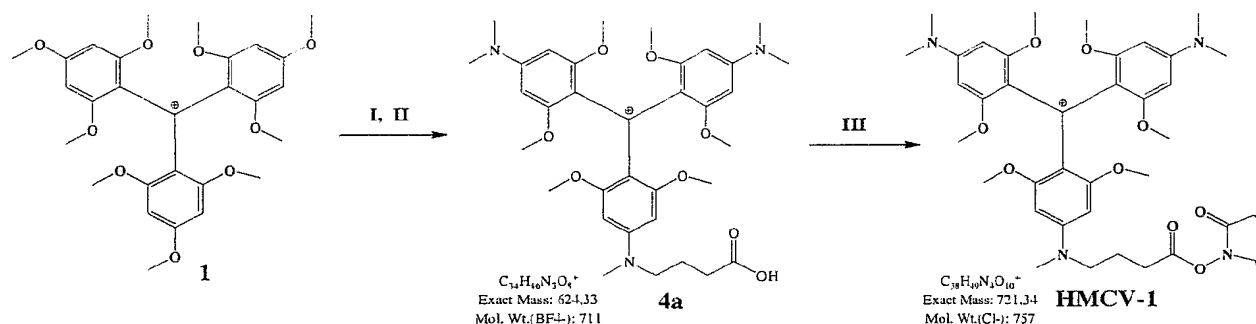
20

Example 3: Preparation of Hexa-Methoxy-Crystal Violet succinimidyl ester (HMCV-1)

**Synthesis of HMCV-1:**

25

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**Scheme 1.** I) 4-(N-methylamino)-butyric acid hydrochloride (1 eq.) Diisopropylethylamine, in acetonitrile, 20 °C, 20 hours. II) Dimethylamine (excess). III) TSTU, Diisopropylethylamine, in acetonitrile, 20 °C, 2 hours.

**4a (BF<sub>4</sub><sup>-</sup>):** 4-(methylamino)butyric acid hydrochloride (1.36 g; 8.8 mmol), **1** (5.0 g; 8.3 mmol), and diisopropylethylamine (5 mL) was dissolved in acetonitrile (120 mL). The reaction mixture was stirred at 30-35 °C in a dry nitrogen atmosphere for 22 h. Aqueous dimethylamine (40 mL of a 40% solution) was added and the reaction mixture was stirred for four more days. Solvent and excess dimethylamine were removed in vacuo and the remaining material dissolved in chloroform. The chloroform solution was washed twice with brine and dried over MgSO<sub>4</sub> before evaporation of the solvent and reprecipitation of the product from CH<sub>2</sub>Cl<sub>2</sub>/ether. Yield: 4.4 g (70%) of a dark blue powder.

MS (FAB<sup>+</sup>): *m/z* 624 (M<sup>+</sup>)

<sup>1</sup>H-NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ 8.34 (1H, bs), 6.03 (2H, s), 5.83 (4H, s), 3.49 (2H, m), 3.46 (6H, s), 3.44 (12H, s), 3.12 (3H, s (masked)), 3.08 (12H, s), 1.94 (2H, t), 1.70 (2H, m).

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**HMCV-1 (Cl<sup>-</sup>):** TSTU (2-succinimido-1,1,3,3-tetramethyluronium tetrafluoroborate; 0.8 g, 2.6 mmol) was added to a solution of **4a** (0.9 g, 1.26 mmol) and diisopropylethylamine (0.55 g, 4.5 mmol) in acetonitrile (15 mL). The reaction mixture was stirred in a closed flask for 2 h, before it was poured into an ice-cold nearly sat. NaCl solution (approx. 150 mL) acidified with HCl-aq (4 mL, 2 M). The water phase was extracted with chloroform (2 x 150 mL). The combined chloroform phases was washed with brine (2 x 50 mL) and dried over MgSO<sub>4</sub>. Evaporation of the solvent and reprecipitation from CH<sub>2</sub>Cl<sub>2</sub>/ether gave a dark blue powder (0.80 g, 84%).

MS (FAB<sup>+</sup>): *m/z* 721 (M<sup>+</sup>)

<sup>1</sup>H-NMR <sup>1</sup>H-NMR br. (400 MHz, DMSO-*d*<sub>6</sub>): δ 5.88 (2H, s), 5.85 (4H, s), 3.60 (2H, s), 3.46 (12H, s), 3.45 (6H, s), 3.15 (12H, s), 3.12 (3H, s), 2.85 (4H, s), 2.80 (2H, t), 1.95 (2H, m).

#### Example 4: Staining of Dextran

70 kDa aminodextran (0.5 mmol NH<sub>2</sub>/g dextran, i.e. 35 moles amine per mole dextran) prepared by an analogous method to that of Example 2 was stained in 10 mM NaHCO<sub>3</sub> pH 8.5, 150 mM NaCl with HMCV-1 (Example 3). The dye was dissolved in dry DMSO and added slowly (10 min.) to the dextran in bicarbonate buffer. Reaction was allowed to take place for 1 hour. The staining was performed with 8 times molar excess of dye. Purification was carried out by dialysis against 10 mM Tris buffer pH 7.4, 150 mM NaCl, 1.25 mM Ca<sup>2+</sup>, 2 mM NaN<sub>3</sub>.



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The obtained degree of labelling of the stained dextran was determined by UV spectroscopy as 7.0 dyes per dextran.

Example 5: Glucose measurement

5

AF594 stained human MBL (Example 1) and HMCV1-Dextran (Example 4) were mixed in TBS buffer (same as above) to concentrations of 10  $\mu$ M of both components (using concentration of MBL-AF594 carbohydrate recognition domains, CRD, each with an Mw of approx 25kDa). The assay chemistry mixture was sucked into a hollow fibre (regenerated cellulose, diameter 0.2 mm).

15 Fluorescence lifetime measurements (frequency domain) were performed in a KOALA automated sample compartment (ISS, Champaign IL). All solutions were pre-heated to 34 °C in a water bath, and all measurements in the KOALA instrument were recorded at 34 °C. The fluorescence cell containing the fibre and fibre-holder assembly was placed in the sample holder of the KOALA, and the fluorescence cell was filled with buffer containing glucose.

25 The measured phase was an average of at least forty phase-angle recordings. After the completion of a measurement, the fluorescence cell was emptied using a pipette, and refilled with buffer containing the next concentration of glucose. A delay of 20 minutes between measurements was introduced to allow the assay chemistry to reach equilibrium.

30 To generate a glucose dose-response curve, the phase was measured at 0, 5, 10, 30, 100 and 500 mM glucose. After

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determination of the phase-angle at 500 mM glucose the fibre was washed several times with 10 mM TRIS buffer over a time period of 60 minutes. At this point the same phase-angle was obtained as for 0 mM Glucose. This demonstrates the

5 reversibility of the assay.

**Table 1** Absolute phase shifts for AF594-MBL and HMCV1-Dex70. The PMT counts reflect the intensity increase of the system.

Glc (mM)	Phase @61 MHz	PMT counts
	10 $\mu$ M/10 $\mu$ M	10 $\mu$ M/10 $\mu$ M
0	36.1	3230
2.5	36.6	3370
5	37.4	3590
10	38.0	4030
25	39.2	4950
50	40.2	5770
500	41.7	7220

10

#### Example 6: Effect of HMCV1-Dextran Molecular Weight

Example 5 was repeated using HMCV1-Dextran of molecular weight ranging from 20 kDa to 250 kDa (prepared in an

15 analogous way to the HMCV1-Dextran used in Example 5). It was found that the highest phase shift was achieved using 110 kDa HMCV1-dextran. The results are shown in Fig. 1.

#### Example 7: Effect of stained MBL:HMCV1-Dextran ratio

20

Example 5 was repeated using a range of ratios of stained MBL:HMCV1-Dextran. It was discovered that a 1:4 ratio of stained MBL:HMCV1-Dextran (5  $\mu$ M concentration of MBL-AF594 carbohydrate recognition domains, CRD, each with an Mw of

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approx 25kDa, and 20  $\mu$ M HMCV1-Dextran, molecular weight 110 kDa) gave an increased response. The results are shown in Table 2.

- 5 **Table 2** Absolute phase shifts for AF594-MBL 1:4 HMCV1-Dex110.

Glucose, mM	Reponse, phase shift
0	0
5	1.4
10	2.6
30	4.8
100	7.5
500	8.1

Example 8: Comparison of Stability of MBL and Con A

10

Example 5 was repeated using MBL-AF594 and ConA-AF594 as lectin with 110 kDa HMCV1-dextran as analyte analogue in physiological TRIS buffer (pH 7.4, sodium, potassium and calcium present in physiological concentrations). The glucose concentration was varied between 2.5, 5 mM, 25 mM and 50 mM in cycles over 12 days. Measurements were taken at 5 minute intervals using a miniaturized time resolved fluorimeter. In the experiment with MBL-AF594 the phase measurements at each glucose level were constant over time. A significant drift was observed in the experiment with Con A, resulting in a more than 10% reduction in the measured phase after 20 days. The results are shown in Fig. 2.

20

Example 9: Preparation of Aminated Periodate oxidised

25

Dextran

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70 kDa dextran (200 mg, 0.00286 mmol) was dissolved in water (2.8 mL) and added to a 100 mM solution of sodium periodate in water (2.8 mL, 100 times molar excess). The mixture was stirred in the dark for 1 h at room temperature. The  
5 resulting mixture was transferred to a dialysis tube (MWCO 10-12 k) and dialysed over night against 5 L water.

After dialysis, the volume was adjusted to 8 ml. The periodate-oxidised dextran was split into two aliquots (4  
10 mL, 100 mg each) and treated for a half hour with 28 % aqueous ammonia (200  $\mu$ L) and benzylamine (300  $\mu$ L) respectively. The imine and iminium derivatives were then reduced with sodium cyanoborohydride (45 mg) overnight at room temperature, and pH around 10.

15

The reaction mixture was dialyzed against  $2 \times 1$  L 20 mM TBS the following day.

The degree of amine incorporation in the periodate oxidised  
20 dextran was determined using elemental analysis.

#### Example 10: Preparation of Mannosylated HSA

4 conjugates were prepared in the following way.

25 To 4 x 2 ml Eppendorf vials were each added HSA (10 mg) dissolved in a 20 mM carbonate buffer (0.4 mL, pH 9.2).  
p-Aminophenyl- $\alpha$ -D-mannopyranosyl isothiocyanate (Man-ITC) (1.6 mL) was added in 15, 30, 60, and 120 molar excess, by preparing four solutions as explained below.

30

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Man-ITC (11.9 mg) was dissolved in DMSO (0.1 mL) and 20 mM Carbonate buffer (3.9 mL, pH 9.2). An aliquot (1.6 mL) of this solution (corresponding to 120 times molar excess) was added to an Eppendorf vial containing HSA (0.4 mL). The rest  
 5 of the Man-ITC solution was diluted to double volume, and from the diluted volume, an aliquot (1.6 mL) was added to another eppendorf vial. This procedure was repeated until the four different HSA:Man-ITC mixtures had been prepared.

10 The four reaction mixtures were incubated in a shaker overnight at room temperature. The resulting glycoconjugates were purified on a PD-10 column. During the purification, the buffer was changed to TBS. The degree of conjugation was determined using MALDI-TOF-MS.

15 **Table 3** Determination of conjugation degree using MALDI-TOF-MS. Peak width estimate was measured around half height. The number of mannose is determined using the following formula:  
 (Peak in MS - 66500) / 313

20

	<i>m/z</i> (MALDI-TOF)	Number of Mannose per HSA.
HSA-Mannose 1:15	67500 - 70000	3 - 11
HSA-Mannose 1:30	67700 - 70600	4 - 13
HSA-Mannose 1:60	68100 - 72300	5 - 18
HSA-Mannose 1:120	68600 - 73400	7 - 22

The different HSA-mannose conjugates have different affinities for MBL.

25

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Example 11: ELLA Assay

Preparation of Biotinylated MBL

Biotin-NHS (20  $\mu$ l, 7 mg/ml in DMSO, ~10-15 eq. per MBL monomer) was added to a solution of MBL (3 ml, 0.53 mg) in PBS (3 mL). The solution was gently stirred for 2 h, then transferred to a dialysis tube (MWCO 10-12K) and dialysed against TBS (2  $\times$  1 L) over the course of 24 h. The resulting biotinylated MBL (0.2 mg/ml) in TBS was used without further purification.

MBL ELLA assay

TBS buffer used in the ELLA assay is 20 mM TRIS, 150 mM NaCl, pH 7.4. 20 mM  $\text{CaCl}_2$  is used where antigen is HSA-mannose and 1.25 mM  $\text{CaCl}_2$  (mimicking physiological calcium concentration) is used where antigen is aminated periodate-treated dextran.

A 96-well microtitre plate was coated, overnight at 5  $^{\circ}\text{C}$ , with two columns of each of the antigens (HSA-Mannose from Example 10, aminodextran, benzylamino periodate-treated dextran from Example 9) (100  $\mu$ L, 20  $\mu$ g/mL) in TBS. Residual binding sites were blocked by the addition of 1 % (w/v) HSA in TBS (150  $\mu$ L). The wells were then washed (2  $\times$  200  $\mu$ L TBS). Dilutions of glucose (from 100 mM to 0 mM) in biotinylated MBL prepared as described above (2  $\mu$ g/mL) were added to a total volume of 100  $\mu$ L. After incubation for 2 h, the plate was emptied and washed (2  $\times$  200  $\mu$ L TBS). Streptavidin-HRP 0.1% (v/v) (100  $\mu$ L) in TBS was added. Following 1 h incubation, plates were emptied, and washed

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(3x200  $\mu$ l TBS). The presence of HRP was visualised by the addition of substrate solution (1 mg *o*-phenylene dihydrochloride) and quenched after 2 min with 1 N sulphuric acid solution. Color development was determined by reading  
5 the absorbance at 490 nm, with background subtraction at 630 nm.

The results are shown in Figs. 3 and 4.

#### 10 Example 12: Co-polymer Synthesis

A water-soluble 40 % Mannose copolymer was prepared by emulsion polymerisation as follows.

To a 250 ml three-necked round-bottomed flask equipped with a mechanical stirrer and a nitrogen tube was added Span80  
15 surfactant (5.7 g; HLB [hydrophile lipophile balance] 4.3, 10% w/w based on toluene), AIBN (30 mg) and toluene (57.3 g). The flask was sealed, purged with nitrogen, and kept under a nitrogen atmosphere throughout the polymerisation. Allyl  $\alpha$ -D-Mannopyranoside (3.52 g), 2-  
20 hydroxyethylacrylate (2.552 g), and *N*-(3-aminopropyl) methacrylamide hydrochloride (0.356 g) were dissolved in water (12.7 g) and filtered to remove insoluble material. This mixture was added to the vigorously stirred mixture in the round-bottomed flask through a rubber septum.  
25 The reaction mixture was stirred at room temperature until a stable emulsion was formed (30 min), then at 60 °C for 4 h. A solution of VA-044 (1 ml, 60 mg/ml) was injected through the septum and polymerisation was continued overnight (17 h). The reaction mixture was cooled to room temperature and  
30 the emulsion was disrupted by the addition of acetone. This caused precipitation of the polymer, which was collected,

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redissolved in water, and precipitated by addition of acetone. The product was dried overnight under vacuum to yield 3.2 g (50 %) crude light yellow polymer. Part of the crude polymer (1.0 g) was dissolved in water (10 ml), and  
5 dialysed (MWCO [molecular weight cut off] 25,000) in water to remove low molecular weight material. Freeze-drying yielded 0.46 g (46 %) fluffy white polymer.

#### Example 13: Staining of Co-Polymer

10 In general the labelling of the co-polymer follows the description provided by Molecular Probes (product information MP00143, Rev. June 2001).

The co-polymer (Example 12) (88.6 mg) was dissolved in 10 mM  
15 NaHCO<sub>3</sub> solution (3 ml; pH 8.5). The polymer solution was divided equally into three Eppendorf vials. HMCV-1 (Example 3) (19.6 mg; 26.1 µmol) was dissolved in dry DMSO (600 µl). The dye was added to the polymer solutions in 10 µl aliquots every 30 seconds, in such a manner that the first vial in  
20 total received 100 µl, the second vial received 200 µl and the third vial received 300 µl. After the addition of the last aliquot, the vials were gently stirred for one hour before the solutions were dialysed (MWCO 10-12,000) in 10 mM TRIS buffer with several buffer changes and until no colour  
25 was visible in the dialysis buffer (usually 6-8 buffer changes of 500 ml and 72 hours).

#### Example 14: FRET assay

Assay chemistry including stained co-polymer solution  
30 (Example 13) (4 µL) and stained MBL solution (Example 1) (8.5 µL) in 10 mM TRIS buffer (12.5 µL) was mixed and



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allowed to stand for at least 1 h after mixing. The assay chemistry was then transferred to a fibre as in Example 5 with a syringe. The fibre was mounted in a custom designed fibre-holder which fitted into a standard fluorescence cell  
5 (10 mm x 10 mm).

Measurements were made as in Example 5 and are shown in Table 4.

10 **Table 4**

Glucose, mM	Response, phase shift
0	0
2.5	1.3
5	2.5
10	4.7
30	9.2
100	12
500	12.5

Example 15: Sensor Formulation and Implantation

15

Fibres were made from 1000PEGT80PBT20 polymer (prepared as described in S. Fakirov and T. Gogeva, Macromol. Chem. 191 (1990) 603-614 with a target of 80 wt% hydrophilic segment and 20 wt% hydrophobic segment) by dipping a glass rod of  
20 diameter 700 µm into a 15% w/w solution of polymer in dichloromethane (DCM) and letting it dry at room temperature. This yielded hollow fibres of outer diameter 900µm with a lumen of diameter 700 µm. The fibre was filled with 5 µM with respect to CRD of AlexaFluor™ stained MBL  
25 (Example 1) and 20 µM of HMCV-1 stained amino-dextran 150 kDa (prepared by an analogous method to that of Example 4). Heating the polymer in order to melt it closed the fibre.

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The welded fibre was tested for leakage before testing and insertion.

5 The glucose response measured by the use of time resolved fluorescence spectroscopy (frequency domain) was as shown in Fig. 5.

10 This type of fibre can be placed in the top of the skin by the use of a needle. A needle of suitable size (large enough to contain the wet fibre) is placed parallel to the skin surface at a depth of approx. 1 mm leaving the needle visible as a shadow through the skin. The fibre (still wet) is placed inside the needle and the needle is removed. Typically no bleeding is observed at the insertion site  
15 after the insertion procedure is completed.

When the fibre is in place the reading device is placed directly above the fibre and the measurements can begin.

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Claims

1. A sensor for the detection or measurement of  
5 carbohydrate analyte in fluid, the sensor comprising  
components of a competitive binding assay the readout  
of which is a detectable or measurable optical signal  
retained by a material that permits diffusion of  
analyte but not the assay components, the assay  
10 components comprising:  
an animal lectin; and  
an analyte analogue capable of competing with analyte  
for binding to the lectin.
- 15 2. A sensor as claimed in Claim 1, wherein the analyte is  
glucose.
3. A sensor as claimed in Claim 2, wherein the analyte  
analogue is capable of competing with glucose at  
20 physiological calcium concentrations.
4. A sensor as claimed in any one of the preceding claims,  
wherein the lectin is a human or humanized lectin.
- 25 5. A sensor as claimed in any one of Claim 4, wherein the  
lectin is derived from the human body or is a  
recombinant lectin.
6. A sensor as claimed in any one of the preceding claims,  
30 wherein the lectin is in multimeric form.

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7. A sensor as claimed in any one of the preceding claims, wherein the lectin is a collectin selected from mannose binding lectin, a pulmonary surfactant protein, conglutinin or collectin-43.
- 5 8. A sensor as claimed in Claim 7 when dependent on Claim 6, wherein the lectin is mannose binding lectin in trimeric and/or tetrameric form.
- 10 9. A sensor as claimed in any one of the preceding claims, wherein the analyte analogue comprises a plurality of carbohydrate or carbohydrate mimetic moieties.
- 15 10. A sensor as claimed in Claim 9, wherein the analyte analogue comprises at least one carbohydrate moiety selected from D-fructose, D-leucrose, N-acetyl-glucosamine, D-mannose, L-fucose, N-acetyl-mannosamine, D-arabinose, myo-inositol, D-tagatose, erlose, D-glucose, D-palatinose, D-turanose, D-ribose,
- 20 D-sorbitol.
11. A sensor as claimed in any one of the preceding claims, wherein the analyte analogue bears one or more energy donor or energy acceptor moieties.
- 25 12. A sensor as claimed in any one of Claims 9 to 11, wherein the analyte analogue is an optionally derivatised polymer of carbohydrate and/or carbohydrate mimetic moieties.
- 30 13. A sensor as claimed in Claim 12, wherein the analyte analogue is selected from optionally derivatised dextran,

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mannan, amylose, amylopectin, glycogen, hyaluronate, chondroitin, heparin, dextrin, inulin, xylan, fructan and chitin.

- 5 14. A sensor as claimed in Claim 13, wherein the analyte analogue is optionally derivatised dextran in which the 3- and/or 4- hydroxyl groups of at least one of the glucose units have been inactivated.
- 10 15. A sensor as claimed in Claim 14, wherein the analyte analogue is optionally derivatised dextran which has been treated with periodate.
- 15 16. A sensor as claimed in any one of Claims 13 to 15, wherein the dextran is aminated.
17. A sensor as claimed in any one of Claims 9 to 11, wherein the analyte analogue is a carbohydrate-protein conjugate or a carbohydrate-dendrimer conjugate.
- 20 18. A sensor as claimed in Claim 17, wherein the analyte analogue is a carbohydrate-albumin conjugate.
- 25 19. A sensor as claimed in any one of Claims 9 to 11, wherein the analyte analogue is a flexible water-soluble non-polysaccharide polymer bearing pendant carbohydrate or carbohydrate mimetic moieties.
- 30 20. A sensor as claimed in any one of the preceding claims, wherein the components of the assay are retained by a shell or matrix material.

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21. A sensor as claimed in any one of the preceding claims,  
wherein the retaining material is biodegradable.

5 22. A sensor as claimed in Claim 21 when dependent on Claim  
20, wherein the components of the assay are retained by  
a shell of biodegradable material encapsulating the  
assay components whilst allowing glucose to contact the  
assay components, wherein the biodegradable material  
10 comprises a co-polymer having hydrophobic and  
hydrophilic units.

23. A sensor as claimed in any one of the preceding claims  
wherein said detectable or measurable optical signal is  
15 generated by proximity based signal  
generating/modulating moieties.

24. A sensor as claimed in Claim 23, wherein the lectin is  
labelled with one of an proximity based signal  
20 generating/modulating moiety pair and the analyte  
analogue is labelled with the other of the proximity  
based signal generating/modulating moiety pair.

25. A method of preparing a sensor as claimed in any one of  
25 the preceding claims comprising one or more of phase  
separation, solvent evaporation, extraction, spray  
drying, spray coating, spray chilling, rotary disk  
atomisation, fluid bed coating, coextrusion and pan  
coating.

30

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26. A method of detecting glucose using a sensor as claimed in any one of Claims 1 to 24, comprising implantation of the sensor into the skin of a mammal, detection or measurement of glucose using external optical means and degradation of the biodegradable material.
27. A method of detecting glucose using a sensor as claimed in any one of Claims 1 to 24, comprising detection or measurement of glucose using external optical means by illumination of a said sensor present in or below the skin of a mammal.
28. A sensor for the detection or measurement of a carbohydrate analyte in fluid, the sensor comprising components of a competitive binding assay the readout of which is a detectable or measurable optical signal retained by a material that permits diffusion of the analyte but not the assay components, the assay components comprising:
- a carbohydrate binding molecule labelled with one of a proximity based signal generating/modulating moiety pair; and
- a carbohydrate analogue capable of competing with the analyte for binding to the carbohydrate binding molecule, the carbohydrate analogue being a flexible water-soluble polymer comprising:
- polymerized residues of monomer units, the first monomer unit residues bearing pendant carbohydrate or carbohydrate mimetic moieties and pendant moieties which are the other of the proximity based signal generating/modulating moiety pair; and/or

P10632WO 07.12.05

co-polymerised residues of first monomer units and  
second monomer units, the first monomer unit residues  
bearing pendant carbohydrate or carbohydrate mimetic  
moieties and the second monomer unit residues bearing  
5 pendant moieties which are the other of the proximity  
based signal generating/modulating moiety pair.



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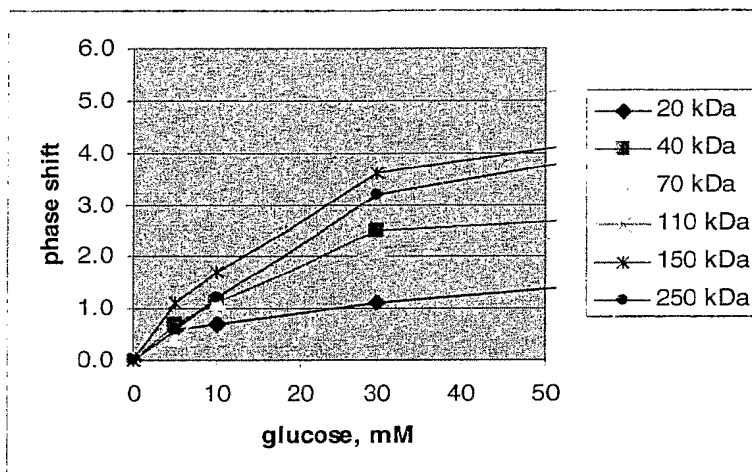
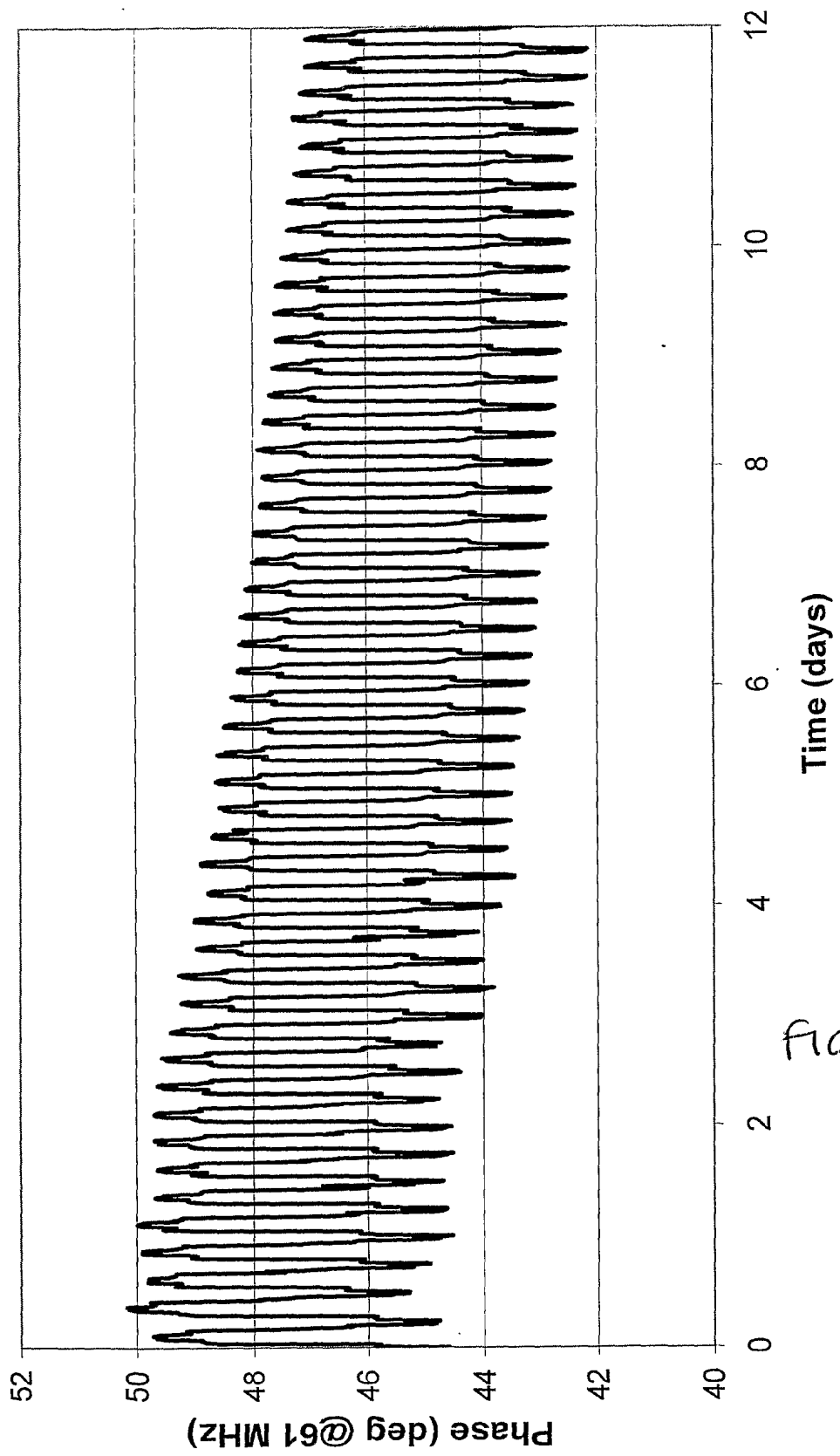
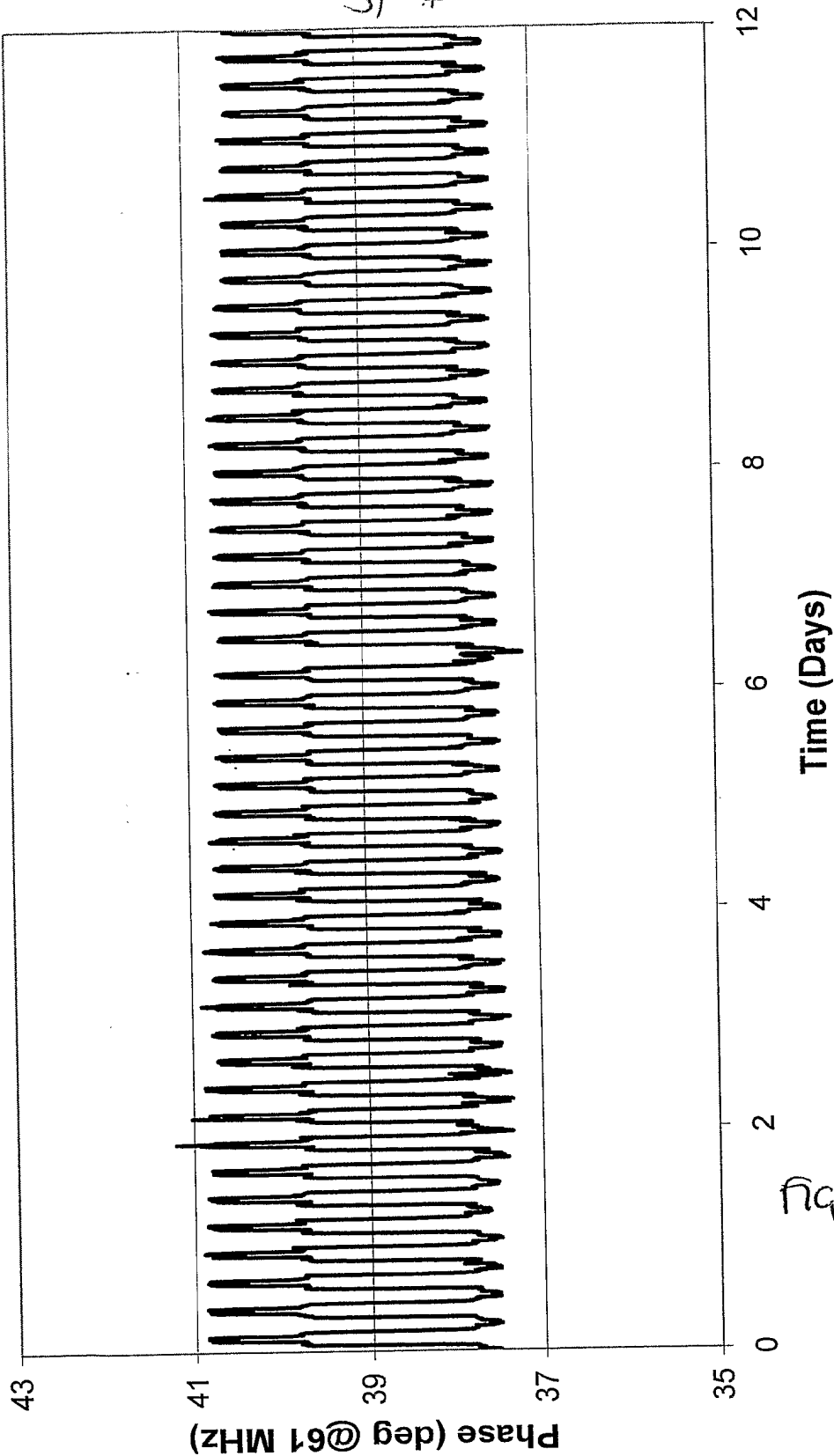


FIG. 1

APD interrogation ConA-Dex



APD interrogation MBL-Dex



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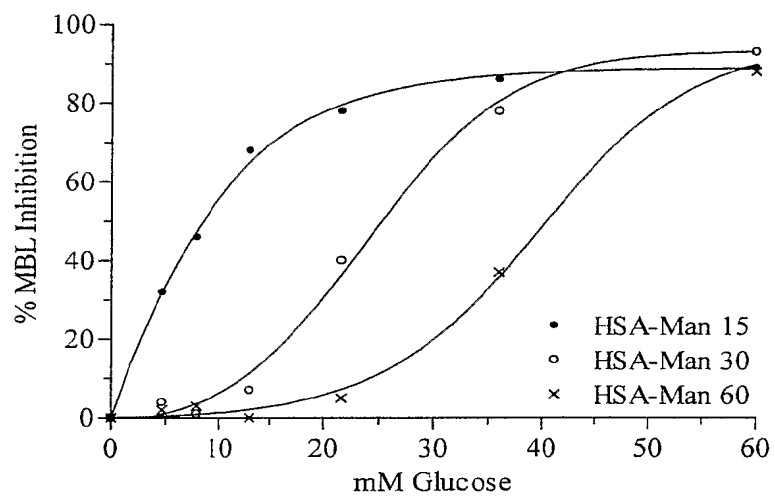


FIG. 3

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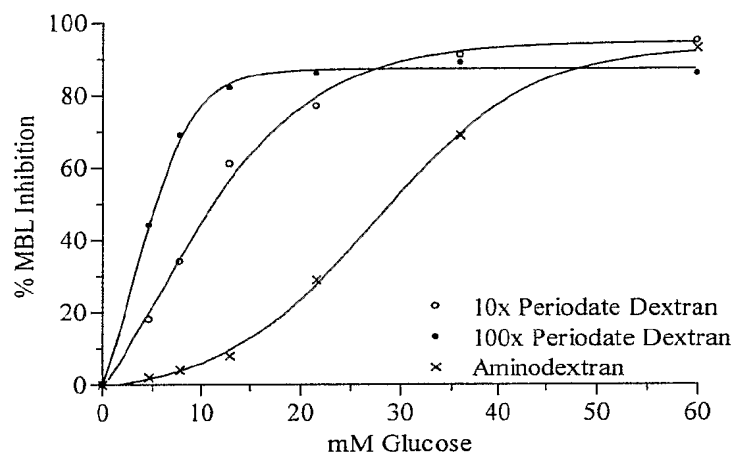
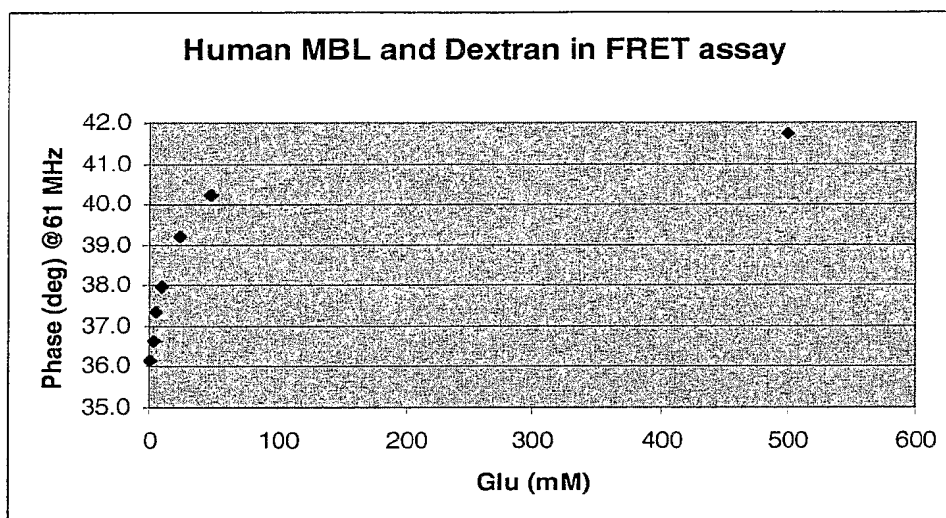


FIG. 4

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**FIG. 5**

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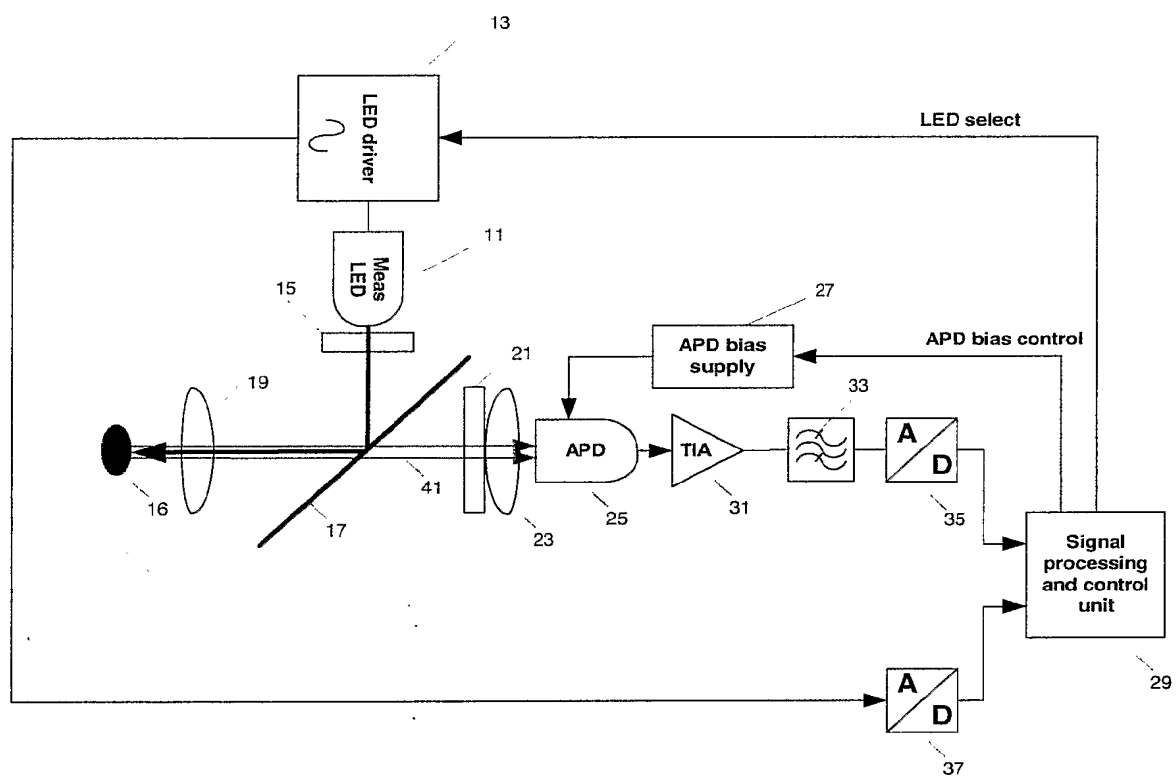


FIG. 6

# INTERNATIONAL SEARCH REPORT

International application No  
**EP2005/013114**

**A. CLASSIFICATION OF SUBJECT MATTER**  
**G01N33/543**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)  
**G01N**

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**EPO-Internal, WPI Data, CHEM ABS Data, BIOSIS**

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BALLERSTADT R ET AL: "COMPETITIVE-BINDING ASSAY METHOD BASED ON FLUORESCENCE QUENCHING OF LIGANDS HELD IN CLOSE PROXIMITY BY A MULTIVALENT RECEPTOR" ANALYTICA CHIMICA ACTA, ELSEVIER, AMSTERDAM, NL, vol. 345, no. 1-3, 1997, pages 203-212, XP000901095 ISSN: 0003-2670 abstract	1,2, 10-14, 20, 23-25,28
A	US 5 342 789 A (CHICK ET AL) 30 August 1994 (1994-08-30) column 4, lines 30-33; example 1	1,2
A	WO 00/16099 A (SENSOR TECHNOLOGIES, INC) 23 March 2000 (2000-03-23) examples	1,2
-/-		

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

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\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*Z\* document member of the same patent family

Date of the actual completion of the international search

**31 March 2006**

Date of mailing of the international search report

**06/04/2006**

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# INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2005/013114

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 98/55869 A (SENSOR TECHNOLOGIES, INC; WOLF, DAVID, E) 10 December 1998 (1998-12-10) examples</p> <p>-----</p>	1,2

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Information on patent family members

International application No

PCT/EP2005/013114

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